Universidade de Lisboa

Faculdade de Medicina

Identification of a transient lymphoid population in the murine epidermis

Francisca Monjardino Ferreira de Almeida

Ramo: Ciências Biomédicas
Especialidade: Imunologia

2015

Universidade de Lisboa

Faculdade de Medicina

Identification of a transient lymphoid population in the murine epidermis

Francisca Monjardino Ferreira de Almeida

Orientador: Dr. Florent Ginhoux
Co-Orientador: Prof. Dr. Luís Graça

Ramo: Ciências Biomédicas
Especialidade: Imunologia

A impressão desta dissertação foi aprovada pelo Conselho Científico da Faculdade de Medicina de Lisboa em reunião de 28 de Abril de 2015.

As opiniões expressas nesta publicação são da exclusiva responsabilidade do seu autor.

This work was supported by FCT under the fellowship SFRH/BD/72641/2010

“Keep your dreams alive. Understand to achieve anything requires faith and belief in yourself, vision, hard work, determination, and dedication. Remember all things are possible for those who believe.”

Gail Devers

TABLE OF CONTENTS

Acknowledgments

First of all, I would like to thank my supervisor Florent who even though has made my life quite challenging throughout the years has always made sure that I would learn new things and would improve as a scientist.
I would also like to thank Luís Graça for accepting to be my co-supervisor, making this move to the other side of the world possible. I also need to acknowledge the members of my committee for the precious feedback during our meetings.

After this incredible emotional rollercoaster there is a great bunch of people that for sure need to be mentioned.
A big thank you to: Naomi for always being there to help, brainstorm or just listen; Donovan for all the mouse help, chocolates and for worrying about me; Peter for the ranting and shared frustration; to Andreas for the good scientific suggestions; Baptiste for showing me that French guys can be nice; Svet for the good laughs at Tea Party; Kazu for the Japanese gifts, good snacks and funny moments; Otsuka for all the help with genotyping and lab stuff in general; Gill for her innocence that always made me laugh; to Mandy for being a great replacement mom and for all the cakes; to Guigui for the great papers!; and to all the other ROs and students that passed by and were part of this journey one way or another.
Thank you SIgN for being more my house than my own apartment!

To Barbora thank you for always having my back and advising me on how to take care of delicate matters. To Leigh for being a great friend ready to listen and give great scientific advice. Carlita for knowing that I could always come to you, in our language, and feel better! To Ross, going through this journey together and sharing the same feelings, I wouldn't even say anything and you would get it immediately!

To the ADS, thank you for being my family when the real one was far away. The memories will never be forgotten and some friendships will never be lost even if we are far away! Being captain of ADS in 2013 will be forever a huge highlight of my stay in SG. Mel, Julie,

Xavier and others thank you for your kind words and preoccupation at times, as well as many laughs and medals!

To Kim for making me want to keep fighting for my dreams. To Leonor for the good dinners and laughs. To Amy for everything: girly talk, dinners, and constant messages. Never thought such a “sport” could lead us so close to each other making you the girlfriend that I have been looking for!

To my 4 families, Monjardino, Ferreira de Almeida, McGuire and Ribeiro da Silva, even far away, by skype, whatsapp or facebook you all have been there for me. A special thanks: to my Grandma that always makes me miss home and her food; to my worried Grandpa that sends me emails with passages from his diary making me go back to my childhood; to my mother for all the weekend chats; to my aunt Luisa that always dropped a message to make sure I am ok; to my uncle Zé e Rita that are always ready to hear me out and advise me on what best to do; to Teresa that worries as she were my own mother and always encourages me to keep going; to my dear cousin Maria that always took my mind of the bad things with her kind words, and to Daniel that put up with me up close, daily, during these last months.

To Mariana and Inês who stretch far beyond their limits in this race against time and gave me great support in order to race side by side with them.

To Marta that has been there for me since my early days in science, ready to listen and advise.

To my babes: Joana and Catarina! You always made me want to dream more and more and never be afraid or give up. Even physically far away I know you are right here with me, no matter where I am. To Nuno for all the incredible support at any time during all these years.

And the most special thanks to Andre, who has taken care of me better than I would ever be able to do on my own. We are in this journey together, and together we shall succeed!

Abbreviation list

AGM - Aorta-gonad mesonephros

BM - Bone Marrow

CCL - CC-Chemokine ligand

CD - Cluster Differentiation

CHILP - Common Helper-like Innate Lymphoid Progenitor

CIP - Committed Intermediate Progenitor

CLA - Cutaneous Lymphocyte-associated Antigen

CLIP - Common Innate Lymphoid Progenitor

CLP - Common Lymphoid Progenitors

cNK - conventional Natural Killer cells

CTP - Committed T cell Progenitor or Circulating T cell Progenitor

DC - Dendritic Cells

DDC - Dermal Dendritic Cells

DETC - Dendritic Epidermal T Cells

dGuo - 2'-deoxyguanosine

DL1 - Delta-like 1

DN - Double negative

DP - Double positive

E - Embryonic age

ELC - Epidermal Lymhoid Cell

ETP - early T lineage progenitors

FTOC - Fetal Thymic Organ Culture

GALT - Gut-associated Lymphoid Tissue

HSC - Hematopoietic Stem Cells

IBD - Inflammatory Bowel Disease

IDC - Interdigitating Cells

IEL - Intraepithelial lymphocytes

IL - Interleukin

ILC - Innate Lymphoid Cells

ILCP - Innate Lymphoid Cell Progenitor

iNK - Immature Natural Killer cell

ISP - Immature Single positive

LC - Langerhans Cells

LTi - Lymphoid Tissue inducer

LTiP - Lymphoid Tissue inducer cell Progenitor

MFI - Mean Fluorescence Intensity

MHC - Major Histocompatibility Complex

NB - Newborn

NK - Natural Killer cells

NKP - Natural Killer cell Progenitor

NSG - NOD scid gamma

PP - Peyer's Patches

pTα - pre-TCRα

Rag - Recombination-activating gene

siLP - small intestine Lamina Propria

SP - Single positive

TCR - T Cell Receptor

T_{EM} - T effector memory cells

T_h - T helper

T_{reg} - Regulatory T cells

T_{RM} - Tissue-resident memory T cells

TSLP - Thymic Stromal Lymphopoetin

WT - Wild Type

YS - Yolk Sac

γ_c - common gamma chain

Figure Index

Introduction and Results

Supplementary figures

Resumo

Linfócitos e o seu desenvolvimento têm sido extremamente estudados nos últimos anos. A família de linfócitos inclui células T, células NK (Natural Killer) e a nova família de ILCs (Innate Lymphoid Cells).

O processo de desenvolvimento de células T a partir de células progenitoras tem sido estudado ao largo de vários anos e é um processo relativamente bem caracterizado: precursores saem da medula óssea ou do fígado fetal, durante o desenvolvimento embrionário, e chegam ao timo como progenitores de células T. Ao longo do desenvolvimento dentro do timo, timócitos imaturos vão adquirindo a expressão de co-receptores e receptores característicos de células maduras, tais como CD3 e TCR (T cell receptor). Ainda que esta seja a rota tomada pela maioria das células T, outros mecanismos geracionais parecem existir tanto *a priori* da migração para o timo, no feto, como independentemente do timo, pós nascimento. Exemplo desta via alternativa é o facto de ratinhos sem timo apresentarem células T nas suas mucosas.

Mesmo dentro das populações de células T que são geradas no timo durante o desenvolvimento embrionário, algumas subpopulações parecem ser capazes de se manterem independemente do timo no adulto, sendo a pele de particular interesse.

DETCs (Dendritic epidermal T cells) são células T caracterizadas pela expressão de Thy1 (thymocyte antigen 1) e um TCR único, Vγ3Vδ1. Estas são as primeiras células a expressarem um TCR no timo fetal e após expressão de CD3 e do TCR maduro esta população expande e migra para a epiderme. Neste tecido, DETCs participam em processos de cicatrização, vigilância imunitária, entre outros. Estudos mostraram que esta população celular tem a capacidade de se renovar independentemente de precursores que circulam no sangue, nomeadamente aquando exposição a radiação. Contudo, o mecanismo exacto pela qual esta renovação ocorre é ainda desconhecido e a questão da existência de progenitores de DETCs na epiderme mantém-se sem resposta.

No laboratório, usando uma combinação de modelos animais imuno-suficientes ou deficientes introduzimos uma nova camada no compartimento linfóide da epiderme. Neste trabalho revelámos a descoberta de uma nova população de linfócitos que aparece na epiderme durante fases tardias do desenvolvimento embrionário e decresce após

nascimento em animais wild type (WT). No entanto, verificámos que esta mesma população de células acumula na epiderme de animais com defeitos intrínsecos ou extrínsecos na geração de células T, tais como ratinhos deficientes em *Rag* ou atímicos. A esta população de células chamámos ELC (Epidermal Lymphoid Cells).

As ELCs apresentam à sua superfície moléculas características de linfócitos, como Thy1 e CD2, contudo não possuem expressão de moléculas características de células T maduras, tais como CD3 ou receptores de células T (TCR) αβ/γδ, sendo este fenótipo característico de progenitores de células T.

De forma semelhante às DETCs, as ELCs mostram ser resistentes a radiação e capazes de se autorrenovarem *in situ*, impondo como possibilidade esta população colonizar a epiderme antes do nascimento. Contudo, ELCs encontradas na epiderme de ratinhos adultos ou na epiderme neonatal apresentam níveis equivalentes de expressão de pre-TCRα (pTα) mas variam nos seus níveis de expressão de CD3 no citoplasma. Tais características estão em concordância com a hipótese preliminar desta população ser um conjunto de progenitores de células T, possivelmente em diferentes fases de desenvolvimento. No entanto, após ensaios de diferenciação *in vivo*, *in vitro* ou *ex vivo*, as ELCs mostraram não ter capacidade de se diferenciarem em células T maduras, ou de progredir no processo de desenvolvimento de progenitores do mesmo tipo celular. Este conjunto de resultados repudiou a hipótese de ELCs serem progenitores de células T.

Uma vez que o antigénio Thy1 não é específico de linfócitos T, sendo também partilhado pela linhagem de células NK, pôs-se a hipótese das ELCs pertencerem à família de células NK. Curiosamente, estas células mostraram ser dependentes de vias de sinalização ligadas a IL-15 e à cadeia gama comum (γ_c). É de notar que todos os linfócitos são dependentes de sinalização através de γ_c e que a interleucina IL-15 tem sido altamente associada ao desenvolvimento e manutenção de células NK. Ademais, ao explorar a expressão de moléculas únicas desta família celular, observámos que as ELCs expressam moléculas como NK1.1, CD49b e CD244 na sua superfície.

Relevante para a caracterização de uma população é o seu perfil de produção de citocinas e de dependência de factores de transcrição. Perante condições estimulatórias as ELCs mostraram-se produtoras de IFN-γ e IL-2. Além disso, análises de animais deficientes para Runx3 e Nfil3, factores de transcrição cruciais para desenvolvimento de

células NK, mostraram um decréscimo significativo na frequência de ELCs nas suas epidermes. Este conjunto de resultados fortalece a possibilidade de ELCs pertencerem à família de células NK.

Em suma este trabalho aponta para a descoberta de uma nova população de linfócitos na epiderme de ratinhos, que devido à fase do desenvolvimento em que são encontrados, poderão interagir com outros componentes celulares e participar em mecanismos tolerogénicos aquando colonização da pele pelo seu microbioma. Contudo, mais estudos terão de ser realizados de forma a compreender a verdadeira natureza, origem e função desta população.

Palavras-chave: linfócitos, desenvolvimento embrionário, epiderme, células T, células NK, progenitores, DETCs

Abstract

T cell progenitors are known to migrate from the fetal liver in embryos and the bone marrow in adults to the thymus where they differentiate into naïve T cells and then egress to peripheral tissues. However, different studies have shown that a pool of T cell progenitors may also exist in the periphery. Here, we identify a lymphoid population resembling these progenitors which transiently seed the epidermis during late embryogenesis in both WT and T cell-deficient mice, which we named ELCs (Epidermal Lymphoid Cells). These $Thy1^{+}CD2^{+}$ cells lack expression of CD3 and TCRαβ/γδ at their surface, reminiscent of the phenotype of extra or intra-thymic T cell progenitors, as well as Natural Killer cells. Similarly to dendritic epidermal T cells (DETC), ELCs are radio-resistant and capable of self-renewal, suggesting that the pool of ELCs that seed the skin during embryogenesis maintains itself throughout life. Additionally, ELCs have partial expression of T cell lineage markers, such as CD3 in their cytoplasm and expression of pTα. However, when introduced to *in vitro*, *ex vivo* and *in vivo* differentiation assays, ELCs did not differentiate into conventional T cells or DETCs, conclusively showing that they are not T cell progenitors. Nonetheless, upon phenotype analysis ELCs revealed to be IL15-dependent, express NK-lineage markers such as NK1.1, CD49b and CD244 and produce IFN-γ and IL-2 under stimulatory conditions. On top of that, ELCs have also shown to be dependent on the transcription factors Runx3 and Nfil3. Altogether, we report the discovery of a unique population of lymphoid cells within the murine epidermis with characteristics that point towards ELCs being a pool of NK-like cells. Whether ELCs interact with other immune or non-immune cell populations leading to unique immune functions in the epidermis remains to be investigated. Moreover, further studies would have to be addressed in order to further characterize the true origin, nature and function of such a population.

Key words: lymphoid cells, embryonic development, epidermis, T cells, Natural Killer cells, progenitors, DETCs

INTRODUCTION

1.Introduction

1.1 Skin as an Immune Organ

As the largest organ of the body, the skin functions as a physical barrier preventing the entry of foreign pathogens; however, at the same time it also provides a home to numerous commensals. The interface between skin microbiota and its derived immunological responses requires a very delicate balance between host and microorganism[1–3]. In mammals, the skin is composed of the epidermis, attached to a basement membrane, underlain by the dermis and a subcutaneous fatty region. The resident cell populations that make up these strata can be broadly divided into immune and non-immune cells. The epidermis is comprised primarily of keratinocytes which are tightly connected to one another forming an outer enucleated, cornified layer referred as the *stratum corneum*. This "brick wall" ensures an effective physical barrier limiting the access to the internal environment[4,5]. The immune pool of the epidermis includes Langerhans cells (LC), Dendritic Epidermal T cells (DETC, restricted to the murine epidermis) and Tissue-resident Memory T cells (T_{RM}) (Figure 1). Of note that T_{RM} colonize the epidermis mostly during inflammatory conditions[6].

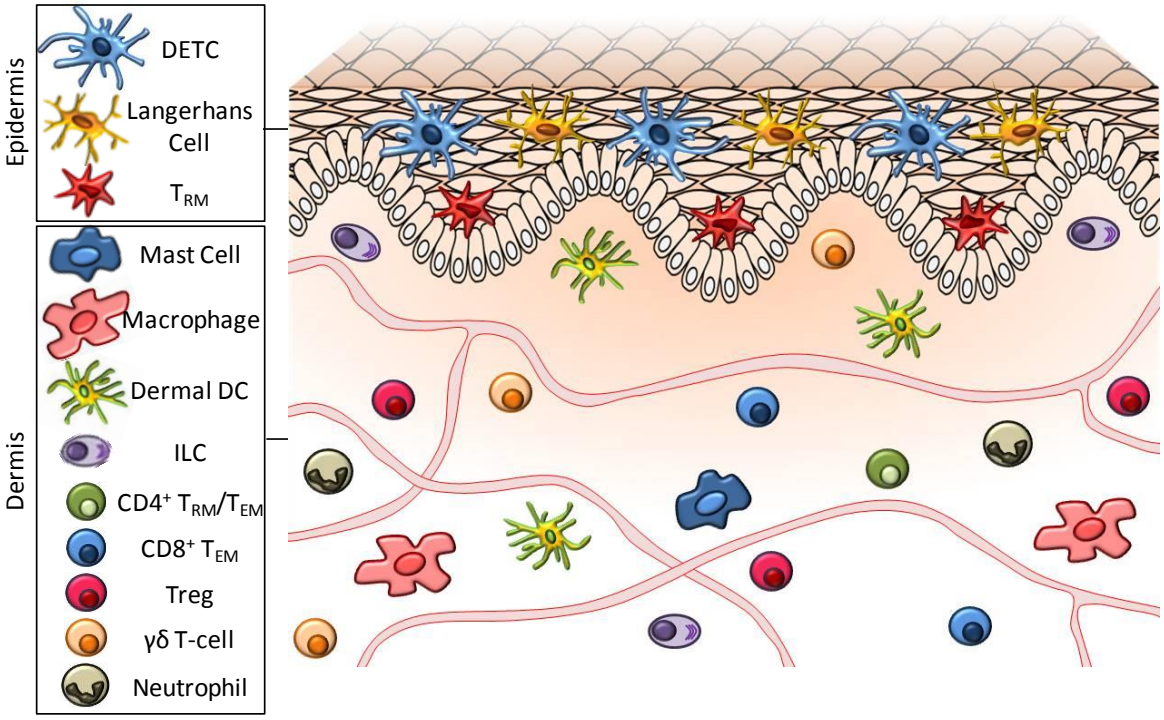


Figure 1. Representative scheme of the skin structure and immune cell types found in the skin.
Skin is composed of epidermis and dermis forming two cellular environments separated by a basement membrane. Multiple immune cell types are found within the skin, including Langerhans cells, Dendritic Epidermal γδ T cells and memory αβ T cells in the epidermis. In steady state, the dermis contains a heterogeneous mix of immune cells, including subsets of dendritic cells, macrophages, neutrophils, mast cells, Innate Lymphoid Cells and T cells. Blood and lymphatic vessels and nerves (not shown) are also present throughout the dermis. Modified from [7].

The epidermis and dermis are physically separated by a thin base-membrane of extracellular matrix proteins that regulates cell movement across. The dermis is primarily comprised of extracellular matrix proteins that give the skin its structure and elasticity. Unlike the epidermis, the dermis allows a less restrained migration of cell populations and has a much more complex cellular constitution. Numerous immune populations can be found in the dermis including dermal Dendritic Cells (DDC), Macrophages, Neutrophils, Mast cells, Innate Lymphoid Cells (ILCs) plus different subsets of T cells (Figure 1) [1,7–9].

1.1.1 Organogenesis

The embryonic epidermis is covered by a transient protective layer of tightly connected squamous endodermis-like cells, known as the periderm. Around embryonic

age (E) 9-12, out of the 20 days of mouse development, and between 4-7 weeks out of the 40 week gestational age (EGA) in humans the periderm appears[10–12]. The periderm first emerges as a simple layer derived from basal keratinocytes, undergoes a series of changes which alter both the surface morphology and the subcellular organization, and then disappears when epidermal keratinization is complete[11]. The function of the periderm is not well described, but may be related to transport/exchange between the fetus and the amniotic fluid. The epidermis loses its periderm while acquiring the *stratum corneum*, which occurs around E16.5–E17.5 in mice and between 15 and 22 week EGA in humans[10,11]. Just below the epidermis is the basal lamina, composed of extracellular matrix proteins including integrin and laminin, followed by the dermis which derives from the mesoderm. The dermis provides mechanical structure via a network of connective tissue, which includes the proteins collagen and elastin. Hair follicles, sebaceous glands, sensory nerves, and vasculature are within the dermis[13].

1.1.2 Other immune sentinels (non-T cells)

Some examples of immune sentinels that can be found in the epidermis and dermis are dendritic cells (DCs), macrophages, mast cells and ILCs.

Skin DCs can be classified according to their location in distinct anatomical compartments of the skin: Langerhans cells are the main DC subset in the epidermis, where they constitutively reside in the suprabasal layers and are regularly spaced among keratinocytes[14]; whereas dermal DCs reside in the dermis immediately below the dermal-epidermal junction. Dermal DCs have been reported to be particularly important for T cell responses and viral immunity[15,16]. Other possible roles are not so clear, though in other tissues they have been linked to T Helper (Th) 17 cell–mediated immunity[17,18].

In the mouse, there are two main DC dermal populations corresponding to the $CD11b^+$ DCs and $CD103^+$ DCs[19,20]. Several studies have reported that $CD11b^+$ DCs can be further divided; with one group deriving from pre-DC and the other derived from monocytes[19,21,22]. The murine skin dermal DC panel can be nicely matched with the DCs in human dermis, which comprises up to three different subsets: $CD1c^+$ DCs, $CD14^+$ DCs and $CD141^+$ DCs subset. Whereas human $CD141^+$ DCs match mouse $CD103^+$ dermal DCs, and

human CD1c$^+$ DCs of pre-DC origin and CD14$^+$ DCs of monocyte origin would together make up the mouse CD11b$^+$ dermal subset [16,23,24]. Langerhans cells (LCs) can be found on both mouse and human epidermis and have been distinguished from other cells by the expression of langerin and CD1a, respectively[16,20]. LCs acquire antigens, transport them to regional lymph nodes, present them to naïve T cells and initiate adaptive immune response[25–27]. LCs have strong immunogenic properties but they may also act as mediators of tolerance, for example to commensal bacteria[28–30]. They are involved in antimicrobial immunity, skin immunosurveillance, induction phase of the contact hypersensitivity and in the pathogenesis of chronic inflammatory diseases of the skin[31,32].

While dermal DCs migrate rapidly to lymph nodes, skin macrophages are predominantly sessile dermal cells[33–35]. Dermal macrophages have been reported to have antimicrobial activity and to produce pro-and anti-inflammatory mediators[36–40]. Mast cells are the major effector cells of immediate hypersensitivity reactions, which are mediated by the release of their granule contents upon antigen-mediated cross-linking of Fc-bound IgE on their surface[41,42]. Similarly to macrophages, mast cells and basophils can have pro-inflammatory and anti-inflammatory functions in the skin[43,44]. Mast cells also seem to have a role in the eradication of parasites, during acute bacterial infections and maybe even in wound healing, but a protective function of mast cells is still under discussion[45,46]. Innate lymphoid cells are a newly described family of cells that have been extensively characterized in the past few years. However, so far only natural killer cells (NKs) and ILC type 2 (ILC2) have been detected in healthy skin. NKs are usually present in small numbers that are increased in inflammatory conditions, such as psoriasis[47–49]. ILC2 have been reported by Roediger *et al* where in the steady state, this population has often been found to associate with mast cells as they trafficked through the dermis. Constitutive production of IL-13 by this ILC2 population has shown to suppress the function of mast cells[50]. However under inflammatory conditions other type of ILCs such as ILC type 3 (ILC3) can be found in the skin[51].

1.1.3 T cells as immune sentinels

In humans, healthy skin contains more than 2x10^{10} skin-resident T cells, which is more than twice the total number of T cells in the blood[52]. The human epidermis harbors

a phenotypically heterogeneous population of T cells, most of which are $CD8^+$ αβ T cells[53,54]. These $CD8^+$ T cells usually reside for longer periods without reentering the circulation, being therefore commonly referred to as tissue-resident memory T cells (T_{RM})[54,55]. Epidermal T cells are mainly distributed in the basal and suprabasal keratinocyte layer, often in close proximity with Langerhans cells[54]. However, in the murine epidermis another population of T cells can be found, the Dendritic Epidermal T cells (DETC). This unique population of γδ T cells forms a prominent network in the skin in mice where they appear to monitor the integrity of the epidermal layer[56–58]. No exact equivalent has been reported in the human so far.

In the dermis, T cells are preferentially clustered around postcapillary venules and are often situated just beneath the dermal-epidermal junction or adjacent to cutaneous appendages[53]. In humans the ratio of $CD4^+$ and $CD8^+$ T cells is generally almost equal and most are memory T cells that express cutaneous lymphocyte-associated antigen (CLA), while in mouse it is believed that the ratio of $CD4^+/CD8^+$ T cells is uneven, as the predominant population in the murine dermis would be $CD4^+$ T cells[52,53,59]. Skin-specific memory T cells gain skin-homing properties after a process known as imprinting, which involves contact with tissue-derived DCs and possibly mesenchymal cells[60,61]. Regulatory T cells (T_{regs}) are also found in substantial numbers in healthy dermis, both mouse and human, and their contribution to immunity of inflammation appears to be regulated by skin commensals[62].

1.1.3.1 γδ T cells

Epithelia exposed to the environment, such as those that line the gastrointestinal, reproductive and bronchoalveolar tracts, as well as skin, are under the surveillance of resident T cells that express mainly γδ invariant or restricted T cell antigen receptors (TCRs). Several studies have reported the variety of γδ T cell in both mouse and human skin but not all of them agree.

As mentioned before, the murine epidermis harbors a very specific type of γδ T cell, DETC. These dendritic shaped T cells have been reported for the first time in 1983 by Georg Stingl's team and after that many other studies have been published where researchers explore the different characteristics of this unique population. DETCs express

a canonical γδ TCR composed of Vγ3/Jγ1-Cγ1 and Vδ1/Dδ2/Jδ2-Cδ chains, and have not been found elsewhere in the adult mouse. DETC precursors are the first T cells to develop in the embryonic thymus, and remain the predominant $CD3^+$ fetal thymocyte until E17.5, coinciding with their migration to the epidermis. DETCs have been reported to work closely with keratinocytes in immunosurveillance of the epidermis. Once keratinocyte distress is detected, DETCs respond by local secretion of chemokines, cytotoxic effector molecules, growth factors and cytokines that orchestrate skin inflammation, tumor killing and wound healing responses[63–65]. While murine epidermal γδ T cells have been extensively studied, dermal γδ T cells have been less well described. However in 2011 Weninger's team has identified an abundant population of resident dermal γδ T cells. These cells can be differentiated from DETCs by the display of a unique phenotypic profile, being $Vγ4^+Vγ3^-$ or $Vγ4^-Vγ3^-$[66]. Both populations are implicated in the immunosurveillance of the skin by being involved in protective immunity against mycobacterial infections and by producing IL-17 upon inflammatory conditions[66,67]. It is possible that murine dermal γδ T cells have other functions that remain to be identified.

On the other hand the majority of T cells in the human skin are far from being γδ T cells. Some studies have reported that around 18-29% of the $CD3^+$ cells of the epidermis express γδ TCR, while others have contradicted these numbers by reporting that less than 0.1% of $CD3^+$ intraepidermal lymphocytes express the γδ TCR[54,68–70]. Characterization of these cells and their role in local immune responses awaits further investigation. In the dermis, human γδ T cells make up a small portion of the total number of T cells. The TCR repertoire of the γδ T cells in the human skin has been described previously as polyclonal, however new studies claim that a much more restricted repertoire is actually present and that is different from the one found in the peripheral blood or in the skin of the mouse[71]. However in general, further studies will be required to better characterize and elucidate the function of γδ T cells in human skin and their involvement in the immunopathogenesis of skin diseases.

1.1.3.2 αβ T cells

Human skin contains a large number of αβ T cells, essentially all of memory phenotype. These memory T cells consist of both $CD4^+$ and $CD8^+$ subset, with about 10%

of the $CD4^+$ T cells expressing Foxp3, indicative of a T_{reg} cell phenotype[72]. Researchers have also classified two major types of αβ T cells: tissue-resident memory cells and effector-memory cells. The first group is disconnected from blood circulation and remains permanently in the tissue, while the second group is in circulation in the blood and just passes through the tissue. Human skin resident T cells have been reported to have a remarkably diverse TCR repertoire, and most have a phenotype of T_h1 effector memory cells, although T_h2 cells, central memory cells, and functional T_{regs} are also present[52].

Carbone's team was one of the first providing evidence that a population of peripheral memory T cells seemed to be in disequilibrium with the circulation in the mouse skin. These resident T cells were $CD8^+$ T cells found preferentially in the epidermis and were expanded in acute infection models[55]. $CD8^+$ T_{RM} are derived from the same precursor cells that give rise to the long-lived memory cells found in circulation. These precursors undergo an *in situ* developmental program that results in their differentiation into specialized, long-lived memory cells adapted to the extralymphoid compartment[6]. Numerous studies have shown that memory T cells in the lung, skin and gut provide increased protection during a secondary infection at these sites[73–75]. Cell migration to the skin is marked by segregation of the $CD4^+$ T effector memory cells (T_{EM}) to the dermis and $CD8^+$ T_{EM} cells to the epidermis[76]. This migration process involves several molecules, where cutaneous effector-memory cells that are programmed to migrate selectively to the skin express P- and E-selectin ligands and the chemokine receptors CCR4 and/or CCR10. These receptors are critical for T cell homing into the skin which interact with the dermal-associated chemokines CC-chemokine ligand 17 (CCL17) and CCL27, respectively. Recent reports have shown that non-haematopoietic peripheral tissue cells and lymph node-resident stromal cells can also influence the tissue tropism of T cells[60,61]. T_{EM} cells generated as a result of epithelial tissue infections can accumulate as T_{RM} cells at both sites of infection as well as at distant sites within the same epithelial tissue, providing broad and long-lived protective T cell immunity against re-infection[77].

1.2 T cell development

Development of mature T cells is a much studied topic, starting from where the progenitors come from before entering the thymus, passing through the different events in this organ and ending with the export of mature naïve T cells to the periphery. However several reports have shown evidence of T cell education occurring outside the thymus.

1.2.1 Hematopoiesis

In adult mammals, hematopoiesis normally occurs in the bone marrow (BM), which supports simultaneously, within distinct cellular environments, the life-long maintenance of stem cells and the regulated production of end-stage lymphoid, myeloid and erythroid cells. Hematopoietic stem cells (HSC) found in the adult bone marrow arise by replication and amplification of a stock of HSC that emerged early in ontogenesis, when the bone marrow had not yet formed. The production of blood stem cells is accomplished by the allocation and specification of distinct embryonic cells in a variety of sites that change during development. In mammals, the sequential sites of hematopoiesis include the yolk sac (YS), an area surrounding the dorsal aorta termed the aorta-gonad mesonephros (AGM) region, the fetal liver, and finally the bone marrow. Recently, the placenta has been recognized as an additional site that participates in hematopoiesis from the AGM to the fetal liver period[78–80]. After E11.5, the fetal liver serves as the major hematopoietic organ generating all hematopoietic lineages[81]. Subsequent definitive hematopoiesis involves colonization of the fetal liver, thymus, spleen, and ultimately the bone marrow[82].

1.2.2 Thymic education

While during development T cell progenitors are released from the fetal liver and into the blood stream making their way into the thymus, during adulthood progenitors are released from the bone marrow instead[83,84]. Once progenitors reach the thymus, they undergo multiple rounds of proliferation and differentiation. This sequence of events leads to T cell lineage commitment, TCR rearrangements, and the generation of αβTCR- or

γδTCR-expressing T cells that function as killers, regulatory cells or producers of specific cytokines[83]. Only after positive selection and maturation are functional T cells released into the periphery or home to the different tissues[85].

Petri's team has shown that an intricate movement through defined thymic regions occurs during thymocyte development. Lymphoid progenitors enter the thymus at the cortico–medullary junction, then migrate to the outer cortex, and finally return to the medulla by a process that includes several carefully coordinated selection events[86–88]. This complex and probably chemokine-directed[89] migration through the thymus has helped establish the view that T cell development requires many unique interactions that can only be provided in the context of the whole thymus.

From the time that a lymphoid precursor arrives in the mouse thymus to the first expression of an αβTCR, it undergoes multiple phenotypically distinct stages that are defined by the expression of CD4, CD8 and other markers. The first stage of thymic differentiation is called Double Negative (DN) 1 defined by the phenotype $CD25^-$ (alpha chain of the IL-2 receptor), $CD44^+$ (Receptor for hyaluronic acid), $CD117^+$ (c-kit, Mast/stem cell growth factor receptor), $IL\text{-}7R\alpha^+$ (Interleukin-7 receptor subunit alpha) and $CD90^+$ (Thy1, Thymocyte antigen 1) (Figure 2Figure 3). The DN1 subpopulation constitutes a heterogeneous mixture of cells, which can be divided into five distinct subsets (DN1a–e) based on surface expression of CD117 (c-kit) and CD24 (heat stable antigen)[90]. Canonical T cell progenitors are confined to the $CD117^{high}$ subsets DN1a and DN1b[90], which correspond to early T lineage progenitors (ETPs), generally considered the earliest intra-thymic T lineage precursors[91–93]. Cells progress from DN1 to DN2 by expressing CD25 (Figure 2Figure 3). At this stage, the first rearrangements start in the β, γ , and δ loci of the TCR; a process that is completed in DN3 cells. DN1 and DN2 thymocytes retain the capacity to differentiate to NK, although a few B and myeloid precursors have been detected[94,95]. Cells then continue to the DN3 stage when they lose expression of CD44 and CD117. The DN3 stage is a mandatory checkpoint at which expression of the pre-TCR or the γδ TCR results in signals that rescue cells from apoptosis and lead to thymocyte proliferation and further differentiation (Figure 2Figure 3). Cells that fail to express a TCR at this stage undergo apoptosis before the transition to DN4 ($CD25^-$)[96].

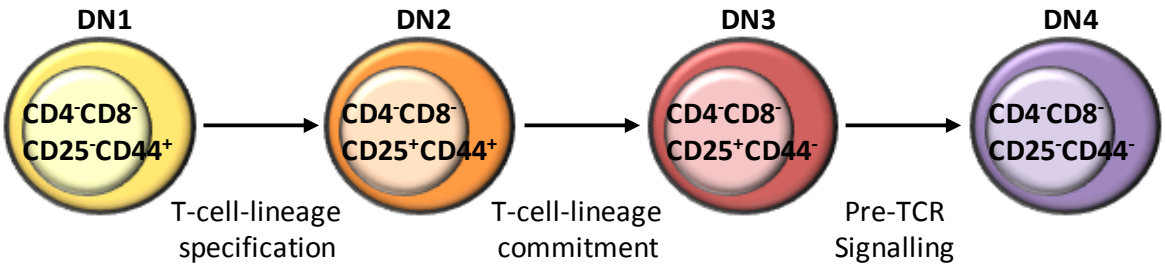


Figure 2. Early stages of T cell development.

The initial stage of T cell progenitor found in the thymus is named double-negative 1 (DN1)–early thymic progenitor (ETP). Upon CD25 expression DN1 transit into DN2, the stage where T cell lineage specification occurs. After the loss of CD44 expression cells become DN3, and the T cell lineage commitment occurs, meaning these progenitors no longer have the ability to differentiate into other cell lineages other than T cell. Followed by the pre-TCR signalling DN3 become DN4 ($CD25^-$), being then ready for a full maturation of the TCR into the next steps of T cell development.

Expression of rearranged TCRβ and formation of the pre-TCR complex leads to a series of events collectively known as β-selection, resulting in allelic exclusion of the TCRβ locus, expansion and differentiation to the double-positive (DP) stage, or γδ selection[97]. As an intermediate stage after the DN stages, thymocytes express CD8 and become immature single-positive (ISP) $CD8^+$ thymocytes before expressing CD4 and becoming $CD4^+CD8^+$ DP thymocytes[98].

During development it takes a little more than a day for the first wave of lymphoid precursors that populate the fetal mouse thymus to generate DN2 cells (this occurs from E12.5 to E14) and only a total of four days for the first DP cells to appear (at E16). By contrast, the lymphoid precursors that continuously enter the thymus throughout young adult life can take ten days to reach the DN2 stage and two weeks to develop into DP cells, with the extra time providing the opportunity for much more extensive proliferation[88,99].

Thereafter, αβ T lineage cells downregulate CD25 and upregulate CD4 and CD8 to generate DP cells that constitute the majority of thymocytes (~80%). DP cells undergo TCRα gene rearrangement and the resulting αβ TCR heterodimer then undergoes Major Histocompatibility Complex (MHC)-mediated selection to yield mature CD4 or CD8 single-positive (SP) T cells. Conversely, most of the γδ T lineage cells remain DN cells but downregulate expression of CD24 following maturation. SP thymocytes have been shown

to reside in medullary areas for up to two weeks, a time during which they undergo changes in expression of a variety of cell-surface molecules, such as CD24, CD62L, CD69, among others, before they are exported to the periphery[100,101]. Interactions between Notch receptor-expressing thymocytes[102] and thymic stromal cells that express Notch ligands[103,104] are also implicated in the complex program of T cell maturation. Ultimately, these interactions result in the generation of self-tolerant $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells, which emigrate from the thymus to establish the peripheral T cell pool.

Interestingly, in addition to the much studied thoracic thymus, a second thymus has been reported in adult mice in the cervical area[105,106]. These cervical lymphoid structures displayed a medulla-cortex architecture, as well as the presence of DP and both $CD4^+$ or $CD8^+$ SP thymocytes with expected levels of TCR/CD3 expression. The cervical thymus appears to support a functional thymocyte development, including selection and export of T cells with diverse TCR repertoire into the periphery. These second thymi appear to arise postnatally, however their anatomic position and number of structures show a degree of variability[105,106]. The actual contribution to the overall T cell pool in the mouse is yet to be clarified. Nevertheless, cervical thymus tissue has been observed in other species, including humans[107]. During embryogenesis, in human fetus, the existence of cervical thymus seems to be quite frequent[108,109]; however their prevalence after birth is usually associated with pathological conditions[110–112].

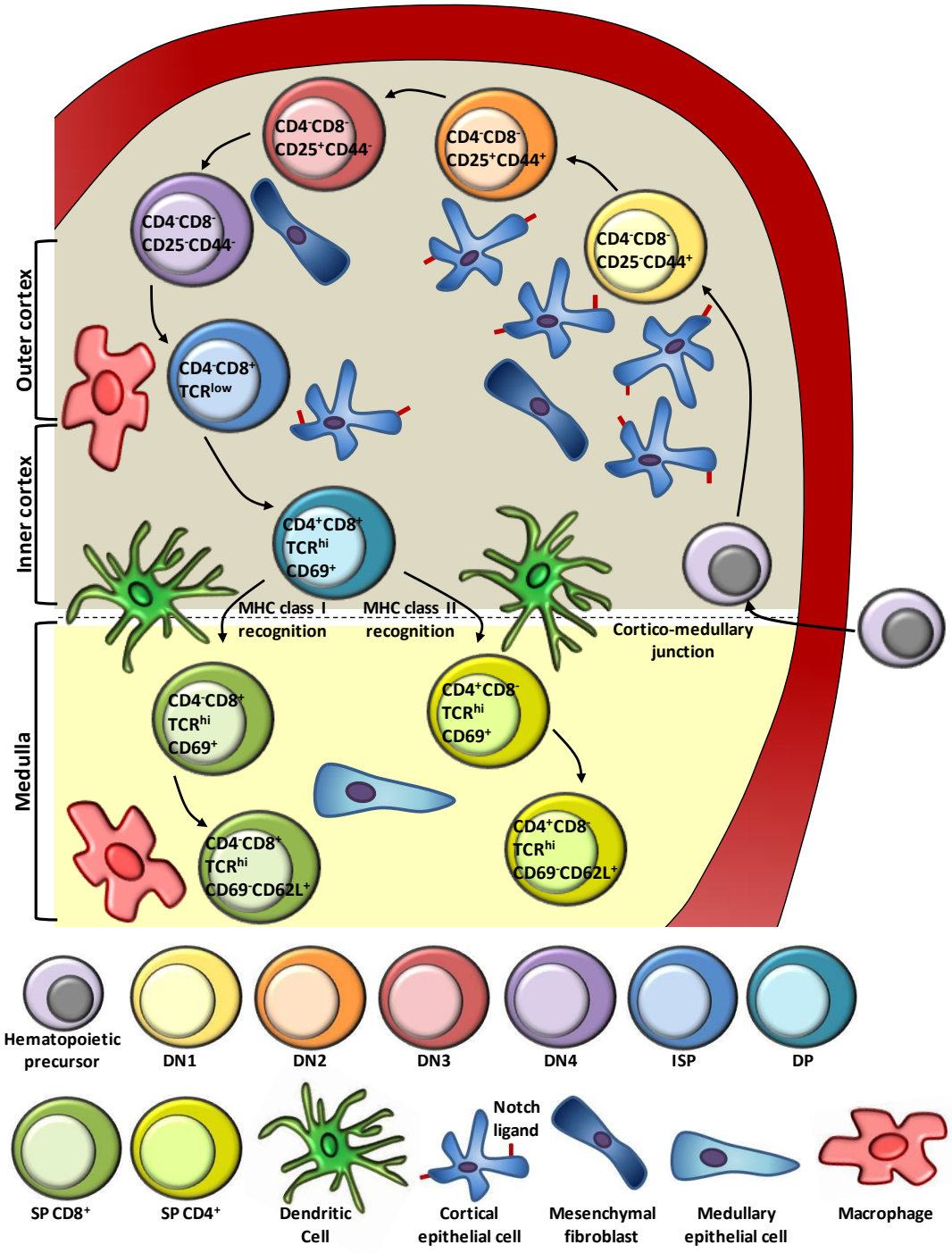


Figure 3. T cell development in the thymus.

The thymus is a lobulated organ divided by mesenchymal septae. Lobes are organized into discrete cortical and medullary areas, each of which is characterized by the presence of particular stromal cell types, as well as thymocyte precursors at defined maturational stages. Thymocyte differentiation can be followed phenotypically by the expression of cell-surface markers, including CD4, CD8, CD44, CD25, CD69 and CD62L, as well as the status of the T cell receptor (TCR). Interactions between thymocytes and thymic stromal cells are known to be important in driving complex programmed T cell maturation in the thymus, which ultimately results in the generation of self-tolerant $CD4^+$ helper and $CD8^+$ cytotoxic T cells. Mature T cells then emigrate from the thymus to establish the peripheral T cell pool. Adapted from [113].

1.2.3 Extrathymic education

Even though thymic development of T cells has been intensely studied in the past years it is still a very controversial field. Several studies have been published showing that extrathymic education of T cells can occur especially since athymic (nude) mice have T cells. This extrathymic lymphopoiesis, however, has special features. It populates mainly the gut mucosa leading to the accumulation of T intraepithelial lymphocytes (IELs). Also in normal euthymic mice, a fraction of T cells do not follow the rules of repertoire selection dictated in the thymus. These cells do not differentiate through the $CD4^+CD8^+\alpha\beta^+$ thymocyte pathway.

1.2.3.1 T cell development in the gut and mesenteric lymph nodes

In wild type mice, the intestinal epithelium harbors different TCR-positive lymphocytes functioning as the first line of defense against enteric pathogens. T-IELs are abundant and consist of $\gamma\delta^+$ T cells (30–40%) and $\alpha\beta^+$ T cells, not only $CD4^+$ or $CD8\alpha\beta^+$ but also $CD8\alpha\alpha^+$, which are a variety of $\alpha\beta^+$ T cells clearly identified only in the gut epithelium[114]. The TCR expression of this last population has a special recognition pattern directed mainly toward MHC I–like antigens[115–117] and also contains superantigen-reactive TCRβ chains[118], suggesting that these cells have not been submitted to negative selection. The likelihood that agonist-selected DP cells may produce a fraction of the CD8αα T-IELs present in normal mice remains to be discussed. CD8αα T-IELs are generated normally in conditions where agonist selection in the thymus is impossible, as in CD3-ζ-deficient mice[119]. Their CD8β chain remains methylated, in contrast to DP cells, and mature $CD4^+$ or $CD8\alpha\beta^+$ cells are present in the periphery or in the gut, indicating that CD8αα T-IELs never went through a DP stage[120]. Finally, the location of DP cells deep in the thymic cortex does not allow them access to efferent capillaries or lymphatics, meaning that DP migration to the periphery is very unlikely to happen[121]. Since they do not appear to belong to the DP main thymic pathway, several research groups called them “thymus-independent”. Nonetheless, experiments with grafts of fetal or neonatal thymus have suggested that at least part of these cells might be of thymic origin[122,123]. All of these observations point towards T-IEL from wild type mice to have a dual thymic and extrathymic origin. It has been proposed that gut cryptopatches, small aggregates of

mucosal lymphocytes at close contact of the epithelium[124], are the extrathymic source of lymphopoiesis contributing to γδ and αβ T IEL in euthymic or athymic mice[125–127]. However, Vassalli's team has reported that extrathymic lymphopoiesis is a minor pathway of T cell differentiation, taking place mostly in the mesenteric lymph nodes and operative only in conditions of defective thymopoiesis but with comparable ontogenic steps[128].

1.2.3.2 Extrathymic T-lineage commitment

The generation of the majority of mature T cells depends on the differentiation of T cell progenitors in the thymus[84]. To ensure this T cell generation, thymus colonization by hematopoietic progenitors is essential. However, it is still highly controversial which progenitors colonize the thymus and where commitment to T cell lineage differentiation occurs. Commitment to lymphocyte differentiation has been associated with the generation of common lymphoid progenitors (CLP) in the bone marrow[129], however several studies have investigated T cell commitment outside bone marrow, before and after birth.

More than twenty years ago Rodewald's team identified a pro-thymocyte in the murine fetal blood, showing that T lineage commitment can precede the thymus development. By *in vivo* transfer these cells could reconstitute the αβ T cell lineage[130]. A few years later Strober and collaborators showed that a microenvironment within the BM is capable of supporting $CD4^+$ and $CD8^+$ αβ T cell maturation from precursors, similarly to the thymus[131]. In addition, Dejbakhsh-Jones and collaborators revealed that an early T cell progenitor could be found in the bone marrow. Through a coculture with T cell depleted BM, they showed that these cells recapitulated the main reported elements of intrathymic T cell maturation, including acquisition of CD2, downregulation of CD16 and CD44, before the appearance of αβ TCR on the intermediary $CD4^+CD8^+$ DP T cells[132]. The same team named Rodewald's cells as Committed T cell Progenitors (CTP)[133] and their recently described bone marrow population as Committed Intermediate Progenitor (CIP), with the second being a possible maturation stage of the first[134]. Furthermore, Krueger and von Boehmer identified a T lineage-committed progenitor in the adult mouse blood that when cultured in OP9-DL1 stromal cells, or upon transfer into *Rag2*$^{-/-}$*Il2rg*$^{-/-}$, generated T lineage cells. They were called Circulating T cell progenitors (also CTP)[135].

In addition to these circulating or BM resident T lineage committed progenitors, others have been reported. Katsura and collaborators have shown that the fetal liver contains progenitors that are selectively potentiated to differentiate into T lineage cells. These were also shown to be distinct from those in the BM, having the ability to generate T but not B or myeloid cells in a much shorter period than BM progenitors[136,137].

Moreover, abundant extrathymic T cell progenitors have been found in athymic mice and following bone marrow transplantation of irradiated mice, possibly as a result of lymphopenia. In addition, upon radiation, major T cell progenitor activity has been reported in bone marrow-derived spleen colonies[138]. Later, the same team provided evidence that this population of progenitors represents a unique population, which is thymus independent and much more efficient at inducing peripheral T cell reconstitution than previously described precursors[139].

Notch signalling has been shown to be active in the extrathymic T lineage-committed progenitors and required for their generation[140]. However, these same progenitors have also been detected in the spleens of unmanipulated wild type mice, indicating that extrathymic T cell commitment can occur under physiologic conditions[141]. The developmental fate of these extrathymic T cell progenitors is not clear.

The origins of extrathymic T progenitors and their relationship to intrathymic progenitors remain to be defined by future studies.

1.3 Natural Killer and Innate Lymphoid Cells

Innate lymphoid cells (ILCs) are emerging as important effectors of innate immunity and have a central role in tissue remodelling[142,143]. ILCs are defined by three main features: the absence of recombination-activating gene (*Rag*)-dependent rearranged antigen receptors; a lack of myeloid cell phenotypical markers; and their lymphoid morphology[144]. The “founding” members of the ILC family are natural killer (NK) cells and lymphoid tissue-inducer (LTi) cells. NK cells mediate early immune responses against viruses and are involved in cytotoxicity while LTi cells are essential for the formation of secondary lymphoid organs during embryogenesis and for postnatal formation of

intestinal lymphoid clusters[145–148]. These cell types were discovered in 1975[149] and 1997[147], respectively, and have been extensively studied throughout the years.

Recently, several distinct ILC populations have been identified that, similarly to NK cells and LTi cells, depend on common gamma chain (γ_c) and inhibitor of DNA-binding 2 (ID2) for their development[150–152]. These ILC populations also rely on signalling through interleukin-7 receptor subunit-α (IL-7Rα) for their development and maintenance. Based on similarities in effector cytokine secretion and developmental requirements, the ILC family can be further divided into type 1, 2 and 3. ILCs are potent innate cytokine producers that respond to changes in the cytokine microenvironment, with demonstrated roles in early infection control, adaptive immune regulation, lymphoid tissue development, and in tissue homeostasis and repair[143,153,154].

1.3.1 Ontogeny

Given that ILCs and adaptive immune system lymphocytes all derive from the common lymphoid progenitor (CLP); the question of whether an ILC lineage-restricted progenitor for some or all ILC populations exists remains unanswered.

Within the bone marrow, the CLP gives rise to all ILC lineages, including B and T cells[142]. The earliest progenitor with restricted lineage potential to all ILCs has recently been identified as the CXCR6$^+$ αLP ($\alpha_4\beta_7$ expressing CLP; also known as the common innate lymphoid progenitor - CILP) (Figure 4)[155]. The CILP shows multi-lineage differentiation, including NK, ILC1, ILC2, and all ILC3 in both *in vitro* and upon transplantation *in vivo*. This progenitor is dependent on Nfil3; consistent with reports showing that *Nfil3*$^{-/-}$ lack all ILC lineages[156,157]. However, group 1 ILC subsets in the salivary glands, uterus, and possibly liver appear to develop independently of Nfil3. Therefore, the CILP may not be completely dependent on Nfil3 or some ILCs may develop from other progenitors.

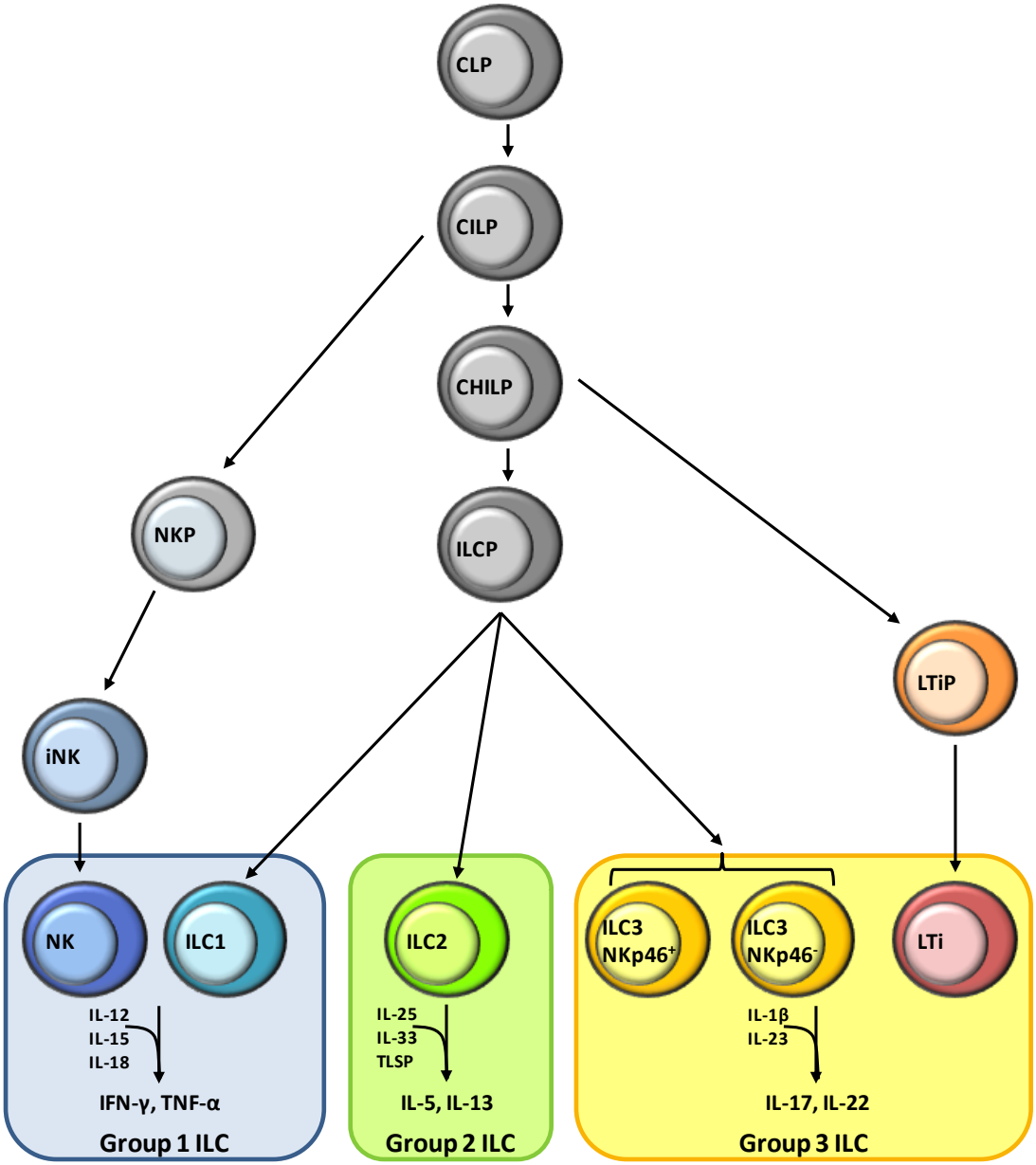


Figure 4. Proposed ILC developmental tree.

All lymphoid lineages are the progeny of the CLP. After the branchpoint with B and T lineages, the existence of an ILC-restricted progenitor (CILP) has been proposed. Downstream two main ILC lineages can be discriminated: killer ILCs and helper-like ILCs. CHILP is the common helper-like innate lymphoid progenitor, where the ability to give rise to EOMES^{+} NK populations has been lost. Killer ILCs are represented by cNK cells and helper-like ILCs are composed of the various cytokine-producing ILC subsets (i.e., ILC1, ILC2 and ILC3). All helper-like ILCs (but not NKs) differentiate from the Id2^{+}PLZF$^{+/-}$ CHILP. This population then bifurcates into RORγt^{hi} LTi precursors (LTiP) and PLZF^{hi} ILCP, the latter of which gives rise to all ILC lineages. NK lineage also has its own skewed progenitors. Transition from CILP to NKP involves a sequential downregulation of CD135, upregulation of CD122, followed by a downregulation of CD127. A sequential population can also be identified as immature NK, where they already express NK1.1 but not CD49b. Acquisition of CD49b is linked to NK maturation. Mature ILC lineages can also be further grouped based on common effector cytokine production, as type 1, 2 or 3 ILC. Adapted from[142,158].

Klose *et al,* by taking advantage of a reporter mouse for the transcription factor Id2, identified a new population of progenitors $Id2^{+}Lin^{-}IL\text{-}7R\alpha^{+}\alpha_4\beta_7^{+}CD25^{-}PLZF^{+/-}$. These cells were named common helper-like innate lymphoid progenitor (CHILP), and identified as the next step in the pathway of ILC development after the CILP stage (Figure 4). Upon *in vivo* transfer, CHILP gave rise to $EOMES^{-}$ ILC1, ILC2, $NKP46^{+}$ ILC3 and $NKP46^{-}$ ILC3, including the LTi subset, but not $EOMES^{+}$ NK cells (Figure 4)[159]. Although the CHILP was identified by Id2 expression, the importance of this transcription factor may not be limited to this population, as the KO model lacks NK cells and all three ILC groups[150,152]. Nevertheless, the CHILP population consisted of a $PLZF^{+}$ and a $PLZF^{-}$ subset, and since PLZF is an Id2-controlled gene, this suggests that the $PLZF^{+}$ subset may be the progeny of $PLZF^{-}$ CHILP[160].

On the other hand and even though progenitors expressed PLZF, Constantinides and collaborators found that ILCs do not express this transcription factor. However, lineage tracing of the expression pattern of *Zbtb16*, the gene encoding PLZF, revealed that all helper-like ILCs were prominently labeled, while conventional NKs (cNK) and LTi were not. As a result, a rare subset of $PLZF^{hi}Lin^{-}IL\text{-}7R\alpha^{+}\alpha4\beta7^{hi}ckit^{+}CXCR6^{-}$ cells was identified in the fetal liver and adult bone marrow. This subset arose as a precursor of ILCs called innate lymphoid cell progenitor (ILCP) (Figure 4). *In vivo* transfer of ILCPs in competition with CLPs showed that the first gave rise to greater frequencies of ILCs, confirming them to be a progenitor skewed towards ILC lineage. ILCPs efficiently generated ILC1, ILC2 and ILC3 but were poor at generating NK cells or LTi[158]. Therefore it is believed that this population is downstream of CHILP (Figure 4). Mice deficient for PLZF have some defects in ILC1 and ILC2, suggesting that it might not be essential for all lineages. On top of that, $PLZF^{hi}$ population has also been described to co-express high amounts of GATA-3, suggesting a link between GATA-3 expression in these ILC precursors and their cell fate potential[158]. Interestingly CLP are negative for GATA-3[161] , leading to the theory that GATA-3 upregulation in CLPs might be involved in the restriction of B lineage fate and thereby generation of T cell and ILC precursors. Such ‘‘bipotent’’ precursors would then further differentiate into more restricted T cell lineage precursors or ILC precursors, where upregulation of Id2 would be a dominant factor in promoting the development of the latter. This model is consistent with the existing data and would clearly distinguish the

developmental pathway of conventional NK cells and LTi (GATA-3 independent) from other ILC subsets (GATA-3 dependent)[162].

Transition from CLP to NK progenitor (NKP) involves a sequential downregulation of CD135, upregulation of CD122, followed by a downregulation of CD127[163–165]. In addition, Id2 expression also helped identifying an early NK cell restricted progenitor, the pre-pro NK. The pre-pro NKs are a transient stage between CLP and NKP where they are $CD135^{-}CD127^{+}$ but negative for CD122[166]. Interestingly, these pre-pro NK cells also appear to phenotypically overlap with an ILC2 precursor population that expresses CD25 and T1/ST2[166,167]. Whether the pre-pro NK population harbors ILC2 potential or whether the described ILC2 precursor contains NK cell potential has not been studied. Potentially, these populations might represent an interface between ILC1 and ILC2 development. Immature NKs (iNKs) are the next step in the development pathway of this specific branch of the ILC family (Figure 4). iNK cells can be identified as $CD3^{-}CD122^{+}NK1.1^{+}CD49b^{-}CD11b^{high}CD27^{low}$ and are thought to represent a transient developmental intermediate that can further differentiate giving rise to mature NK cells ($NK1.1^{+}CD49b^{+}CD11b^{low}CD27^{high}$) that circulate in the blood and are detected throughout the body[150,168]. It is worth mentioning that the complex process of NK lineage differentiation can occur at several distinct tissue sites, including bone marrow, liver, thymus, spleen and lymph nodes, and may involve the circulation of NK cells at different stages of maturation between tissue[169]

The T-box transcription factors T-bet and Eomes have important roles in NK cell development. Mice deficient in either of these T-box transcription factors show substantially impaired NK cell numbers and diminished function of splenic NK cells, which is further aggravated by the combined deficiency of the two factors[170,171]. Interestingly, the transcription factor expression of Eomes in immature NK cells coincides with the appearance of $CD49b^{+}$ (i.e. mature) NK cells. Eomes seems to be specifically required for this maturation step in the bone marrow as conditional *Eomes*-deficient mice have a developmental arrest at the $CD49b^{-}$ stage. Consequently, these mice virtually lack all $CD49b^{+}$ NK cells in spleen, liver and lymph nodes and harbour only low numbers of immature NK cells in the periphery[171].

Recent work has demonstrated that other IFN-γ-producing $NK1.1^{+}$ cells are present in mucosal sites in humans[172] and in mice[159,173]. These ILC1 subsets require the

transcription factors T-bet, Nfil3, and GATA-3 for their generation and are phenotypically distinct from conventional NKs. The molecular mechanism through which GATA-3 contributes to ILC1 development or whether its maintained expression is needed for functional attributes is unclear.

GATA-3 transcriptional activity is a major determinant of ILC2 cell fate in uncommitted lymphoid precursors. The transition from CLP to ILC2 is associated with upregulation of Id2 and RORα, both of which are essential for ILC2 differentiation[167,174].

ILC3s are the most complex group of ILCs, all of which strictly depend on the transcription factor RORγt[175]. More recently, GATA-3 was shown to be crucial for development of both LTi cells and T-bet$^+$ ILC3[176,177]. ILC3s express abundant GATA-3 protein, albeit in lower amounts than observed in mature ILC2s[176,177]. Moreover, in the fetus, GATA-3 is critical for differentiation of CD135$^+\alpha_4\beta_7^+$ CLP-like cells and cell-intrinsic GATA-3 expression is essential to generate fetal liver RORγt^{hi}IL-7Rα^{hi} precursor cells (LTiPs) (Figure 4)[176]. LTi, which are closely related to RORγt$^+$ ILC3, derive from $\alpha_4\beta_7^+$ precursors, while the other ILC3 derive from $\alpha_4\beta_7^-$ precursors that express low levels of RORγt[175]. Interestingly, Id2 is required for the generation of $\alpha_4\beta_7^+$ precursors, but not for the generation of $\alpha_4\beta_7^-$RORγt^{low} precursors, demonstrating that $\alpha_4\beta_7^+$ precursors of LTi cells do not generate the precursors of the other ILC3. Nevertheless, all ILC3 fail to develop in *Id2*-deficient mice[152,178]. Another study has potentially identified LTiP as a distinct population consisting of RORγt^{high}PLZF$^-$ cells, which was more abundant in the fetal liver, and co-expressed high levels of TOX but not GATA3[158]. Cherrier *et al* have shown that Notch signaling and Id2 are sequentially engaged to generate $\alpha_4\beta_7^+$ precursors and that Notch signaling must then be terminated to up-regulate RORγt and generate LTi cells[179].

1.3.2 Phenotype, localization and function of ILC and cNK

Mature ILCs have been extensively studied in both mouse and human in terms of surface marker expression, tissue localization and function. ILCs can also readily respond to a vast array of cytokines such as IL-1, IL-12, IL-7, IL-23, IL-25, IL-33 and thymic stromal lymphopoietin (TSLP)[180]. Recent research suggests that ILC populations have important effector functions during the early stages of immune responses against

microorganisms[151,181], they also contribute to tissue repair[182,183], to the anatomical containment of commensal microorganisms[184] and the maintenance of epithelial integrity at barrier surfaces[185,186]. However, ILCs can also cause inflammatory disorders if improperly activated[171,187,188]. In addition, ILCs have also shown to play a relevant role in organogenesis of lymphoid organs in both embryonic development or during adulthood[147,189,190]. Therefore, it is believed that ILCs are exquisitely sensitive to environmental cues and continuously adapt to rapidly changing settings, such as the ones present at mucosal surfaces.

1.3.2.1 cNK

In humans, cNKs include the $CD56^{bright}CD16^{-}$ and the $CD56^{dim}CD16^{+}$ subsets present in peripheral blood. $CD56^{bright}$ NK cells are specialized in IFN-γ secretion in response to DC/Macrophage-derived cytokines, such as IL-12 and IL-18[191] or T cell-derived cytokines, such as IL-2[192], a functional feature that places them close to other ILC1 subsets. $CD56^{dim}CD16^{+}$ NK are specialized in cytotoxicity, since they can readily release lytic granules containing perforin and granzyme upon contact with sensitive targets. However, it has been shown that $CD56^{dim}$ NKs can also produce IFN-γ, although with a more rapid kinetic and in a less sustained fashion than $CD56^{bright}$ NKs[193]. In addition, it has been suggested that $CD56^{bright}$ can differentiate into the $CD56^{dim}$ subset upon activation[194,195]. $CD56^{bright}$ cells produce additional cytokines such as GM-CSF, TNF-α, IL-13 and IL-10, suggesting that they may have an immunoregulatory function in specific circumstances[196].

In the mouse, cNKs include mature and immature circulating splenic and bone marrow NKs[197], $CD127^{+}$ IL7-dependent thymic-derived NKs[198], and different subsets of tissue-resident NKs[199,200], whose nature, function, and relationship to other emerging subsets of ILC1 are just beginning to be understood. Tissue NKs include an abundant population of salivary gland NKs, which are poorly cytotoxic and low producers of cytokines[201,202], liver-resident $CD49a^{+}CD49b^{-}$ NK cells, and skin- and uterus-resident NK cells[203]. Thymic NKs, for their ability to produce IFN-γ, TNF-α, and GM-CSF in response to IL12, are thought to represent the murine counterpart of human $CD56^{bright}$ NK cells. One

unifying feature of all NK cell subsets, including thymic NKs[204], is their dependence on IL-15 and IL-15Rα for development, survival, and maintenance.

1.3.2.2 ILC1

The identification of *bona fide* ILC1 in mice was obscured by the fact that ILC1 were found to express NK receptors such as natural killer cell p46-related protein (NKp46) and NK1.1, which have served as an operative definition of NK cells[173]. Likewise, the same has been found in human with ILC1 expressing CD56, NKG2D, NKp46 and C-type lectin CD161, typical NK markers[172].

Nevertheless genetic reporter systems for lineage-defining transcription factors have allowed us to identify intestinal ILC1 as an ILC lineage separate from cNK cells (expressing Eomes reporter) and NKp46-expressing ILC3 (expressing Rorc reporter)[159].

Two major ILC1 subsets have been identified in human: one present in mucosal tissues expressing CD127 and CD161, but not expressing other markers of the NK lineage[172]; plus another population present in tonsillar tissue and characterized by the expression of several NK-related markers such as CD56, NKp46, and NKp44[173]. However the latter also expresses markers of tissue-resident memory CD8 T cells such as CD103, CD49a, and CD101.

Although the function of these newly identified subsets of ILC1 have not been thoroughly investigated, they may play important pathogenic roles in human inflammatory bowel diseases (IBDs) as both $CD56^{-}CD127^{+}$ and $NKp44^{+}CD103^{+}$ ILC1 are increased in patients with Crohn's disease, as compared to control individuals[172,173].

Mouse equivalent subsets have also been found. A potential murine equivalent of the $CD56^{-}CD127^{+}$ human ILC1 has recently been described also in the small intestine lamina propria (siLP). These cells are $CD127^{+}$, but also express markers of the NK lineage, such as NKp46 and NK1.1. Interestingly, siLP ILC1, like NKs, depend on IL-15, but not IL-7, for their development[205,206]. *In vivo* these cells are major producers of IFN-γ and TNF-α in response to oral infection with *Toxoplasma gondii* and promote clearance of this pathogen by recruiting inflammatory monocytes[159,205]. Under these conditions siLP ILC1 play a major protective role. In addition, the same authors that reported the tonsillar ILC1

in humans have also reported an equivalent population present in the mouse small intestine. Murine ILC1 have an intraepithelial location and are distinguished by the expression of CD160. Intraepithelial ILC1 are distinguished by cNKs due to the fact that they do not respond to IL-12 and IL-18. Alternatively, they secrete large amounts of IFN-γ upon stimulation with IL-15. Notably, and contrary to NK, intraepithelial ILC1 are only partially dependent on IL-15/IL-15Rα signalling for their development. Interestingly, these ILC1 seem to promote tissue damage in a mouse model of colitis induced by CD40 ligation in immune deficient mice[173].

Another subset of ILC1 under debate in the past few years is the so called "ex-RORγt ILC3". *In vitro* approaches have shown that ILC3, in response to cytokines such as IL-15, IL-2, IL-12 and IL-23 that induce IFN-γ production, can be converted into ILC type 1[172,207]. These ILC1 can also be induced *in vivo* by transfer of RORγt^{+} ILC3 and visualized by fate-mapping experiments in siLP[159,208,209].

1.3.2.3 ILC2

ILC2 are the most homogeneous ILC class, expressing largely conserved markers in all tissues, such as IL-7Rα, CD25, Sca-1, KLRG1, IL-33 receptors ST2, among others[153].

ILC2 are dependent on the transcription factors GATA-3[151] and RORα[167,174], TCF-1 and Notch[210] for their development. Functionally, ILC2 are well known to produce IL-5 and IL-13 in a GATA-3 dependent manner[177], as well as IL-4[211]. ILC2 also produce IL-9, and notably, IL-9/IL-9 receptor signalling is required for their survival[212].

ILC2 can be found in various tissues including adipose tissue-associated lymphoid structures[151], gut[213], lung[214] and, as recently described, the dermis[50,215,216]. They promote expulsion of parasites[217,218] and maintain lung homeostasis[183] or drive airway hyper-reactivity during viral infections, such as influenza[219]. ILC2 also contribute to the pathogenesis of atopic dermatitis[50,215,216]. Also in the skin, ILC2 rapidly respond to the alarmin IL-33, to the IL-17 family member IL-25 and to TSLP[50,215,216]. In visceral adipose tissue, ILC2 maintain metabolic homeostasis by recruiting eosinophils, which sustain macrophage alternative activation[220,221]. In addition to type 2 cytokines, ILC2 produce amphiregulin and support the recovery of epithelial barrier integrity after tissue damage[183].

In humans ILC2 express CD161 and high levels of the prostaglandin D2 receptor CRTH2[222]. Notably, ILC2 are highly enriched in nasal polyps of patients with chronic rhinosinusitis[222], suggesting that they might play a fundamental role in human T_h2-mediated diseases such as asthma and atopic dermatitis.

1.3.2.4 ILC3

ILC3 are a highly complex group and include many reported subsets in adult mice, as well as additional fetal and neonatal LTi subsets that have been recognized as key factors for lymphoid organogenesis[190].

ILC3 are grouped together based on shared expression of RORγt, but vary in their expression of T-bet, cell-surface markers, and cytokine production profiles. In the adult, ILC3 can be found mainly in mucosal tissues, such as the small intestine and large intestine, Peyer's Patches (PP), and gut-associated lymphoid tissue (GALT)[47,181,223–225]. Small numbers of ILC3 are present in the spleen[226] and lung[227]. Curiously, the first ILC3 to be characterized was in human tonsils[181,223].

ILC3 subsets include four populations found in greater frequencies in the siLP: $CD4^+$ and $CD4^-$ subsets of $CCR6^+NKp46^-Ror\gamma t^+LTi$[175], $NKp46^-Ror\gamma t^+T\text{-}bet^+$ ILC3 progenitors[208], and $NKp46^+Ror\gamma t^+T\text{-}bet^+$ Notch-dependent ILC3[208,228–230]. One additional, and very controversial, subset of IL-17 and IFN-γ producing $Ror\gamma t^+NKp46^-$ ILC3 is present in the large intestine[231].

Similarly to ILC1, group 3 ILCs are particularly complicated due to evidence of diverging progenitors for different subsets that share many of the same functions and cell surface markers. A simplified model of differentiation proposes that there is progressive differentiation from $NKp46^-$ ILC3, to $NKp46^+$ ILC3, and finally to $NKp46^+NK1.1^+$ ex-Rorγt ILC3[153]. In contrast, $CCR6^+NKp46^-$ LTi do not give rise to $NKp46^+$ ILC3 after transfer[208]. Recent gene expression profiling demonstrated that $CD4^+$ and $CD4^-$ $NKp46^-$ LTi subsets have minimal transcriptional differences and are unlikely to be functionally distinct[232]. However, the developmental relationships between the different subsets within the ILC3 group will require further experimental approaches.

Generally, ILC3 produce the Th17 signature cytokines IL-22 and/or IL-17[47,181,224,233,234]. IL-22 is a member of the IL-10 family of cytokines[235], which acts through an IL-22 dimeric receptor only expressed by non-immune stromal cells[236]. Recent studies have described that IL-22 mediates barrier integrity during homeostatic conditions and mucosal remodeling during injury and infection. In the intestine, ILC3 can produce IL-22 upon stimulation with IL-23 produced by inflammatory monocytes and $CD11^+$ conventional DCs[237,238]. The IL-22 produced by ILC3 is absolutely required for immunity to attaching-and-effacing bacterial infections[239], and together with IL-18 has demonstrated novel antiviral functions[240]. Nevertheless, IL-22 signaling can also be pathogenic in certain circumstances, leading to the secretion of neutrophil chemoattractants by epithelial cells and dysregulated neutrophil recruitment[241]. In addition, while controlled IL-22-mediated survival and proliferation of epithelial cells may favor tissue healing and repair, prolonged IL-22 signaling, and sustained epithelial proliferation may drive tumor formation[242,243]. Notably, tumorigenesis may also be increased by ILC3-produced IL-17, as loss of IL-17 in *Rag*-deficient mice diminishes adenoma development[244]. ILC3 have also been involved in other human diseases, such as Crohn's disease[245] and psoriasis[246].

In addition to sensing cytokines released in the surrounding microenvironment, ILC3 are also sensitive to nutrients. Recent work has shown that vitamin A deficiency results in decreased numbers of ILC3 in the intestine, which increases susceptibility to bacterial infections[247]. Moreover, vitamin A intake by pregnant mothers controls the pool of $CD4^+$ LTi in the embryos, the size of the lymph nodes and PP, and the efficacy of immune responses to viral infections[148].

AIMS OF THE THESIS

2. Aims of the thesis

Lymphoid populations have been thoroughly studied in the skin under both steady state and inflammatory conditions. DETCs are the major T cell population to exist in the murine epidermis in steady state[56]. However during or after inflammatory conditions, resident $CD8^+$ T cells can be found preferentially in the epidermis[55]. Yet, when looking into the dermis, other lymphoid cells can be found such as αβ T cells, NKs and the recently described ILC2[1,50]. Here, we have identified, in newborn and adult mice, a new lymphoid population present in the epidermis.

In this thesis, we aimed at characterizing this newly discovered population, named Epidermal Lymphoid Cells (ELCs). In detail the aims are:

1. To understand the nature of this population, having as possibilities all classes of lymphoid cells (T cells, ILCs or NKs)
2. To characterize their phenotype, transcription factor requirements and their possible function through cytokine production
3. To investigate their developmental and homeostatic cues

Taken together, the ultimate goal of this thesis is to understand the true potential of this population demonstrating that DETCs are not the only lymphoid population present in the epidermis in steady state conditions. Highlighting the presence of another lymphoid population in the epidermis may reveal helpful insights in understanding the immune tolerance of the newborn to the establishment of the microbial community.

MATERIAL AND METHODS

3. Material and Methods

Mice

C57BL/6 (CD45.2) mice (WT) were purchased from the Biological Resource Center (BRC), Agency for Science, Technology and Research (A*STAR), Singapore. C57BL/6 (CD45.1), B6.Cg-*Foxn1*^{nu}/J (Nude), B6.129S7-Rag1tm1Mom/J (*Rag1*$^{-/-}$), B6.129P-Cx3cr1tm1Litt/J mice (*Cx3cr1*$^{gfp/+}$), B6.129P2(Cg)-Rorctm2Litt/J (*Rorc*$^{-/-}$) were purchased from the Jackson Laboratory (Jackson Laboratory, Bar Harbor, USA). B6.129S6-Rag2tm1Fwa N12 (*Rag2*$^{-/-}$) were purchased from Taconic (Taconic Farms, USA). *Rag1*$^{-/-}$ mice were crossed with C57BL/6 CD45.1 to give rise to congenic *Rag1*$^{-/-}$ CD45.1 mice as well as with *Cx3cr1*$^{gfp/+}$ mice to create *Rag1*$^{-/-}$*Cx3cr1*$^{gfp/+}$ mice. NOD *scid* gamma (NSG) were kindly obtained from SIgN Mouse Core facility. *Runx3*$^{-/-}$ mice were kindly obtained from Dr. Motomi Osato, National University of Singapore. All mice were bred and maintained in the SIgN animal facility, and analyzed at late embryogenesis (E17.5 and E18.5), newborn stage, 6 days after birth and adult at 8-12 weeks old (wo). All experiments and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of A*STAR (Biopolis, Singapore) in accordance with the guidelines of the Agri-Food and Veterinary Authority (AVA) and the National Advisory Committee for Laboratory Animal Research (NACLAR) of Singapore. All the *Il15Ra*$^{-/-}$ mice were kindly provided by Dr. Melanie Greter and bred at Institute of Experimental Immunology, Department of Myeloid Cell Immunology, University of Zurich, Switzerland. All the pTα-fate mapping mice[248] were kindly provided by Dr. Hans Jörg Fehling and bred at the Institute of Immunology, University Clinics Ulm, Germany. Both *Tbx21*$^{-/-}$[249] and *Nfil3*$^{-/-}$[250] mice were kindly provided by Dr. Laura Mackay and bred at the University of Melbourne, Australia.

Flow cytometry, cell sorting and intracellular cytokine staining

Flow cytometry was performed on an LSR II 5 lasers and Aria II 4 or 5 lasers (Becton Dickinson, San Jose, USA) and analyzed with FlowJo software (Tree Star, Ashland, USA). Fluorochrome-conjugated monoclonal antibodies (mAbs) specific to mouse CD45 (30F11), CD45.1 (A20), CD45.2 (104), CD2 (RM2-5), CD3 (145-2C11), CD4 (GK1.5), CD8 (53-6.7),

CD48 (HM48-1), CD49b (DX5), CD90.2 (53-2.1), TCRβ (H57-597), TCRγ/δ (GL-3), Vγ3 (536), CD103 (2E7), CD244 (244F4), CD278 (C398.4A), NK1.1 (PK136), IL-2Rβ (TMb1), IL-2 (JES6-5H4), IFN-γ (XMG1.2), IL-13 (eBio13A), TNF-α (MP6-XT22) were purchased either from BD Biosciences (San Jose, USA) or Ebiosciences (San Diego, USA). DAPI labeling was used to exclude dead cells. Intracellular labeling for CD3, IL-2, IFN-γ, IL-13 and TNF-α was performed on cells previously labeled for surface markers, following fixation and permeabilization with Fix/Perm solutions (BD Biosciences, Mountain View, CA) according to the manufacturer's instructions. Intracellular cytokine staining was performed on epidermal cell suspensions after stimulation for 4 hr with phorbol myristate acetate (PMA) (500 ng/mL) and ionomycin (500 ng/mL) (Sigma-Aldrich). For the last 2h of stimulation Brefeldin A (1 ng/mL) (Sigma-Aldrich) was added to the stimulated wells 2h after PMA and ionomycin was added.

Mouse skin cells preparation

Mouse skin cells were isolated as described previously[251]. Briefly, embryonic/neonatal skin was detached from the body or adult mouse ears were split into dorsal and ventral halves and floated in RPMI-1640 medium (Sigma-Aldrich) containing 1 mg/ml dispase (Invitrogen) for 60 min to allow separation of epidermal and dermal sheets. Epidermal and dermal sheets were then cut into small pieces and incubated in RPMI containing 10% fetal calf serum (FCS), 0.8 mg/ml collagenase type IV (Sigma-Aldrich) and 50µg/ml DNase I (Roche) for 90min. Cell suspensions were then syringed through a 19-gauge needle and filtered through 70µM cell strainer (BD Falcon) to obtain a homogeneous single cell suspension. For purpose of cell number normalization, analyses were performed on both ears of the adult mice and the corresponding area, $3cm^2$, of the body skin from the embryos and newborn mice.

Intravital multiphoton imaging of mouse ear skin

Mice were anaesthetized with a cocktail of 150 mg/kg ketamine and 10 mg/kg xylazine, before ear hair was removed with depilatory lotion Veet. Anaesthetized mice were immobilized on a custom-made stage[252] with a heating pad attached to maintain animal temperature at 37°C. To label blood vessels *in vivo*, mice were retro-orbitally injected with 40 µl of 10 mg/ml of Evans Blue dye (Sigma-Aldrich). Images were acquired using a

multiphoton microscope system (LaVision Biotec) with a tunable Chameleon Ultra II Ti:Sapphire laser (Coherent) at 950 nm, and the following long pass mirrors and bandpass filters: 495 LPXR (Chroma), 560 LPXR (Chroma); 475/42 (Semrock) , 525/50 (Chroma), 655/40 (Semrock). Data sets generated were analyzed by IMARIS image analysis software (Bitplane).

Generation of bone marrow chimeras

Recipient 7 to 8 week old (wo) CD45.2 *Rag1*$^{-/-}$ were lethally irradiated (2x 600 rad, 3 hours apart using a Cesium source) and reconstituted by intravenous injection with 1x10^{6} cells of CD45.1 WT BM. Engraftment was assessed by measuring the percentage of donor cells among blood CD45^{+} cells 4 weeks after transplantation. Adult recipients were analyzed two or six months post-transplant. Proportion of DETCs, ELCs and ILCs derived from WT (CD45.1) cells was determined.

Generation of parabiotic mice

CD45.2 *Rag1*$^{-/-}$ mice were sutured to either CD45.1 WT or CD45.1 *Rag1*$^{-/-}$ and were left together for four months before analysis. All mice were 5 to 6 wo at the time of surgery. Mice received 150 mg/kg ketamine and 10 mg/kg xylazine as an anesthetic plus buprenophine and baytril were injected intradermal for a few days after surgery.

Generation of *Rosa26-Rag* mice for inducible Rag1/2 activity

In order to generate a mouse line in which *Rag1* and *Rag2* expression can be induced from the Rosa26 locus by removal of the STOP cassette upon Cre expression, we used 2A peptides[253] for bicistronic expression. We amplified mouse *Rag2* coding region from mouse genomic DNA by PCR reaction with primers (Rag2-5' and Rag2-2A-3') which allowed us to added 2A peptide sequences after the stop codon for *Rag2* translation. We also amplified *Rag1* cDNA by PCR. These PCR products were cloned into the pCR-TOPOII vector (Invitrogen), and their sequences verified. We then inserted *Rag2-2A* fragment in front of *Rag1* cDNA fragment in pCR-TOPOII, followed by preparation of an entire *Rag2-2A-Rag1* fragment by NotI digestion and a ligation into the NotI site of pCTV vector (Addgene) (**Figure S2a**). The linealized targeting vector was transfected into M1 embryonic stem (ES) cells by electroporation. After G418 selection, ES clones that

underwent homologous recombination were screened by PCR as previously described[254]. Appropriate ES cell clone was used to generate the chimera mice.

Primers: Rag2-5' (5'- CGGCGCGCC AGCATAATTACCAATATGAAAAGATATTC -3'), Rag2-2A-3' (5'- CGGATCCCCTGGGCCAGGATTCTCCTCGACGTCACCGCATGTTAGCAGACTTCCTCTGCCCT CTCCACTGCCATCAAACAGTCTTCTAAGGAAGGATTTC-3'), Rag1-5' (5'-GGATCCTATGGCTGCC TCCTTGCCGTCTACCCTGAGC-3') and Rag1-3' (5'- CGGCGCGCCATGTGGAGATCCTATTTAAAA CTCCATTGA -3').

***In vitro* recombination assay**

To measure Rag1/2 activity from *Rosa26-Rag1A2* locus that was induced upon Cre expression, we used a recombination template vector, pJH200[255]. Embryonic feeder cells generated from E13.5 embryos, which harbor WT or heterozygous for *Rosa26-Rag* alleles, were transfected with pJH200 with or without Cre expression vector, pMC-Cre, by FuGENE reagent (Promega) (**Figure S2b**). Three days after transfection, cell lysates were prepared and were analyzed for *Rag*-dependent recombination events by PCR as previously described[255].

Stromal Culture

Stromal cell culture methods were modified from[256]. In brief, TSt4/DL1 cells (kindly obtained from Dr. Ikawa, RIKEN Institute, Japan) were co-cultured for 9-12 days with purified ELCs, from WT newborn epidermis, in the presence of 10ng/ml IL-2, 10ng/ml IL-7, and 10ng/ml IL-15 (R&D systems), in RPMI media supplemented with 10% FCS, 2mM L-glutamine, 1mM sodium pyruvate, 2mg/ml sodium bicarbonate, 0,1mM nonessential amino acids, 50 µM 2-mercaptoethanol, 100 U/ml penicillin, and 100 mg/ml streptomycin.

Fetal Thymic Organ Cultures (FTOC)

The experiment was performed as described[257]. In summary, 2-deoxyguanosine-treated E15.5 fetal thymic lobes from CD45.1 WT were cultured for 7 days before reconstitution with purified ELCs from CD45.2 WT newborns. Around 10 to 15x10^3 ELCs were added to the thymic culture by the hanging-drop technique over 24h and then cultured for 12 to 15 days. RPMI-1640 medium (Sigma-Aldrich) supplemented with 10% of Hyclone FCS (GE

Healthcare Life Sciences), 5µM 2-mercaptoethanol, 10 mM Hepes, 2mM L-glutamine, 100 U/ml penicillin and 100 µg/streptomycin was used throughout the culture. Medium was additionally supplemented with 1.35mM of 2'-deoxyguanosine (dGuo, Sigma-Aldrich) to treat the E15.5 thymic lobes. Treatment with dGuo selectively eliminates thymocytes, interdigitating cells and DCs from the thymic cultured tissue, allowing the introduction of new hematopoietic progenitors to the fetal thymic stromal environment.

NanoString

Direct mRNA expression levels of the samples were measured using the NanoString nCounter gene expression system. 6-10x10^3 cells in 5uL of RLT buffer were hybridized with probes from the nCounter Mouse Immunology panel V1 at 65^{o}C for 17 hours according to the nCounter™ Gene Expression Assay Manual. Post-hybridization processing were done using the nCounter Prep Station (GEN1) and the nCounter Digital Analyzer (GEN1). Four lanes of NanoString data were obtained as RCC files and processed using BIOVIA Pipeline Pilot. The geometrical means of the positive control probe counts were computed for each lane and a scaling factor computed for each lane being the average of the geometrical means of all lanes divided by the geometrical mean of that particular lane. This scaling factor was then applied to all probe counts for all lanes as a means to normalize for the technical variability of the platform. The house keeping genes *Cltc*, *Gapdh*, *Gusb*, *Hprt*, *Pgk1* and *Tubb5* were then used to normalize for any RNA loading differences. This was performed in the same manner as the positive control probes where the scaling factor was computed from the geometrical mean of the house keeping genes. The positive control and housekeeping normalized counts were then logarithmically transformed and used for all subsequent analysis. Data visualization was performed using TIBCO Spotfire. Threshold was set at 100 average counts to define high expression, ten times higher than the control.

Statistics

Statistical analysis was performed on GraphPad Prism6. Mann-Whitney tests were performed using unpaired experimental designs with non-parametric tests. Significance was defined at $p<0.05$ (ns $p>0.05$, * $p\leq0.5$, ** $p\leq0.01$, *** $p\leq0.001$ and **** $p\leq0.0001$). Unless specified all error bars in graphs represent Standard Error of the Mean (SEM).

RESULTS

4. Results

4.1 Murine epidermis contains a population of Thy1$^+$ cells that are distinct from DETCs

The mouse epidermis has two major hematopoietic populations under steady state conditions, DETCs and LCs, which together with keratinocytes work together towards immune defense of the skin.

The *Rag*-deficient models lack all T and B cells[258,259], including DETCs, being therefore believed to be depleted of any lymphoid population in their epidermis. However, when analyzing the Thy1 expression in the epidermis of wild type (WT) and *Rag*-deficient models we identified a significant population of Thy1$^+$, CD3$^-$ and TCRβ$^-$ cells (Figure 5a). Even though these cells were present in the WT epidermis, they existed at much lower frequencies compared to the accumulated population found in the *Rag*-deficient models. Curiously, this population expressed CD103, an integrin broadly expressed by intraepithelial lymphocytes[260–262]. However, while the fractalkine receptor CX_3CR1 was also expressed at high levels by 100% of these cells in the *Rag*-deficient animals, in the minor Thy1$^+$ population from the WT, expression was variable and restricted to a fraction of the cells (Figure 5b).

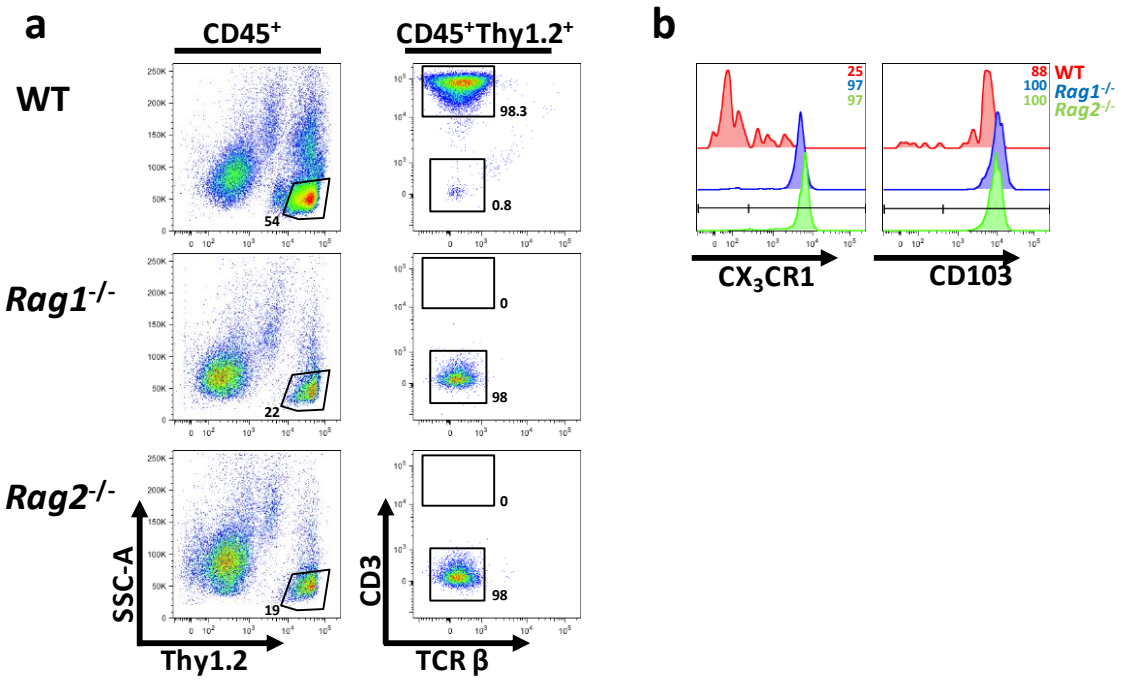


Figure 5. Thy1$^+$ cells are present in the *Rag*-deficient epidermis.

Epidermal cell suspensions from 8wo mice were analyzed by Flow Cytometry. **a.** Representative plots to identify a Thy1$^+$CD3$^-$TCRβ$^-$ population in WT and both *Rag*-deficient models are shown. **b.** Histograms show relative expression of CX_3CR1 and CD103 among Thy1$^+$CD3$^-$ cells. Representative data from n>5

Using the *Cx3cr1*$^{gfp/+}$ reporter model, we investigated their cell distribution in the *Rag1*$^{-/-}$ epidermis. In the WT, DETCs are positive for CX_3CR1 and therefore these mice exhibited a uniform network of GFP$^+$ cells. Interestingly, the *Rag1*$^{-/-}$*Cx3cr1*$^{gfp/+}$ epidermis revealed only a few clusters of GFP$^+$ cells, having also patches that were completely lacking any CX_3CR1 (Figure 6a). We hypothesize that these clusters might be the result of clonal expansion of different progenitors that arrived in the skin.

While investigating their cell morphology and sphericity, we observed that Thy1$^+$CD3$^-$ cells in the *Rag1*$^{-/-}$ are more spherical than WT DETC (Figure 6b and c). Such detailed analysis of the morphology of the WT Thy1$^+$CD3$^-$ population was not possible due to the fact that these cells are rare, have a heterogeneous expression level for CX_3CR1, and do not express any specific marker that would allow us to distinguish them from DETCs.

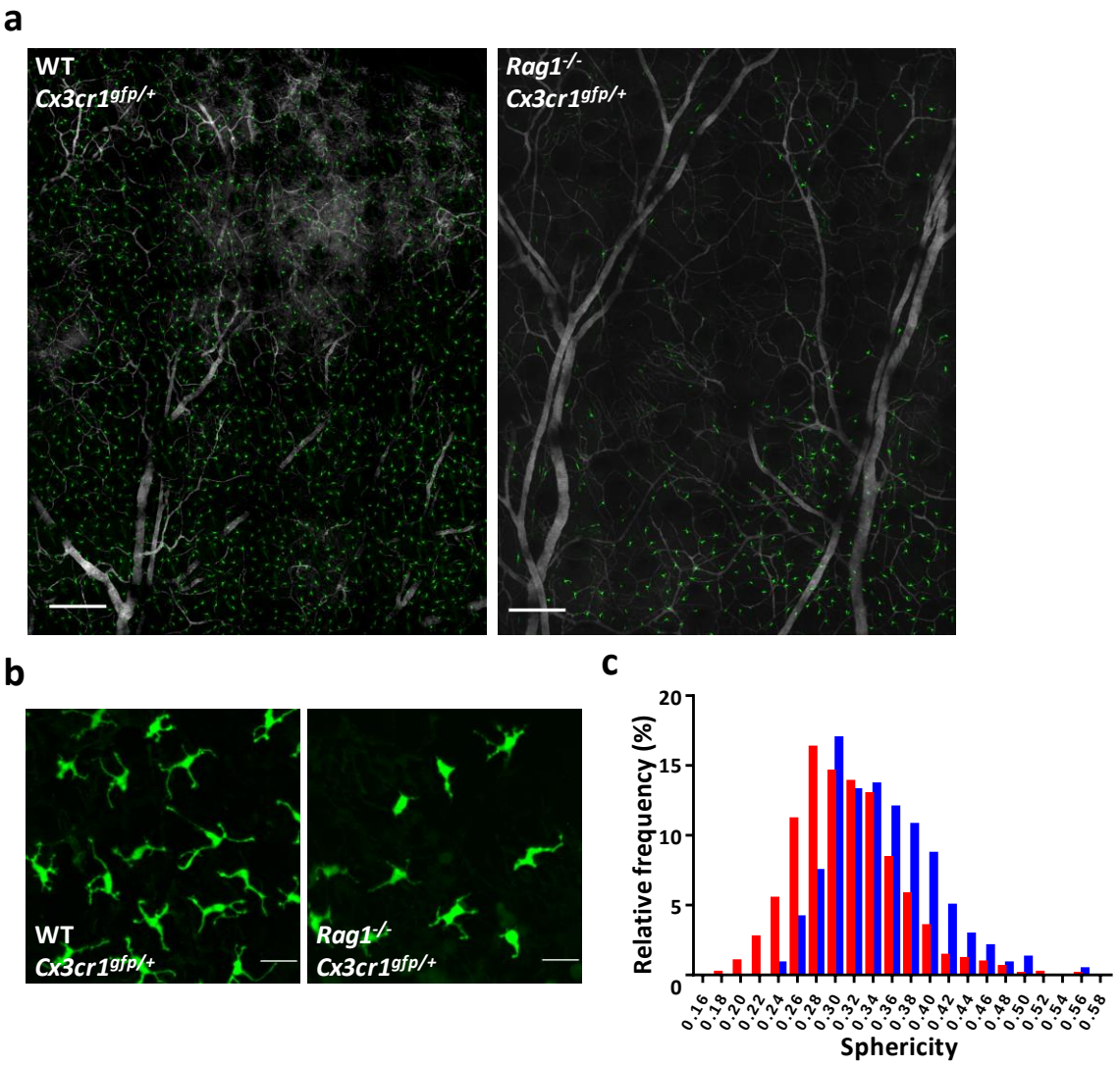


Figure 6. Thy1^{+} cells are present in the *Rag1*$^{-/-}$ epidermis.

a-b. Two photon imaging of the epidermis of WT and *Rag1*$^{-/-}$*Cx3cr1*$^{gfp/+}$ mice. In green are the CX_3CR1-positive cells and in white the blood vessels, scale 200µm (a) and 20µm (b). **c.** Cell morphology analysis of CX_3CR1-positive cells from WT (red) and *Rag1*$^{-/-}$ mice (blue). Representative data of n=3.

4.2 Differential CD2 expression sub-divides the minor epidermal Thy1^{+} population

ILCs are part of the lymphoid family that expresses the Thy1 molecule. Thus we questioned whether the newly identified Thy1^{+} population could be part of this family of cells.

ILCs are known to have morphological characteristics of lymphoid cells yet lack the rearranged antigen receptor. In addition, ILCs can be divided into three main groups based on the transcription factors involved in their development, as well as their produced cytokines[180,263,264]. Analyses of peripheral tissues have shown that ILCs are spread throughout the body, being more prominent in mucosal areas that harbor more than one group of ILC[153].

Recently, ILC type 2 has been described in the dermis, as Thy1^{hi}CD3^{-}CD2^{-}ICOS^{+}[50], a similar phenotype to epidermal Thy1^{hi}CD3^{-} cells. Using the same gating strategy as Roediger *et al*[50] (Figure S1), we identified in both *Rag1*$^{-/-}$ and WT dermis two populations expressing high levels of Thy1 and negative for CD3: a CD2^{+} (blue) and a CD2^{-} (red). However both populations were positive for ICOS (Inducible T-cell COStimulator). Nevertheless, the same populations could be found in the epidermis of both WT and *Rag1*$^{-/-}$ mouse models (Figure 7a). In contrast to what we observed in the dermis, while the minor CD2^{-} population was positive for ICOS, likely corresponding to ILC2; the major CD2^{+} population was negative for ICOS (Figure 7a). Thus, the majority of the novel Thy1^{+} population that expresses the intercellular adhesion/T cell activation molecule CD2, and lacks ICOS, is distinct from both DETCs and ILCs in the murine epidermis. Thus we conclude that these cells represent a novel and discrete population, which we have termed Epidermal Lymphoid Cells (ELCs).

Upon these new results we can conclude that both WT and *Rag1*$^{-/-}$ epidermis harbour a fraction of CD3 negative cells that include ELCs which express high levels of CX_3CR1 and ILCs that have a heterogeneous profile of the same fractalkine receptor (Figure 7b).

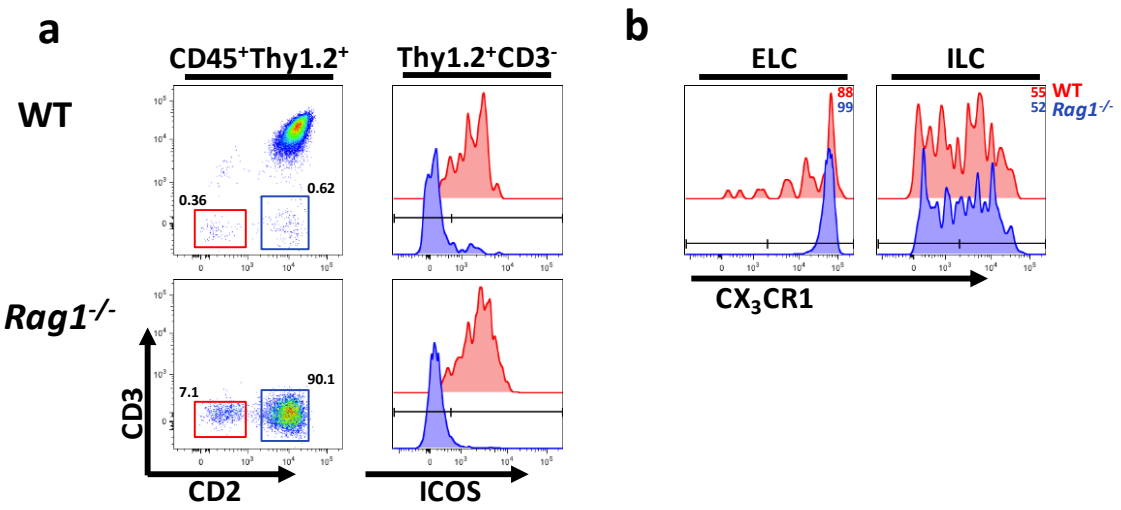


Figure 7. Thy1$^+$ population is heterogeneous.

a. Representative flow cytometry plots and histograms used to identify ICOS expression over the Thy1$^+$CD3$^-$CD2$^-$ (ELC, red) and Thy1$^+$CD3$^-$CD2$^+$ (ILC, blue) populations in both WT and *Rag1*$^{-/-}$ models are shown. **b.** Histograms show relative expression of CX_3CR1 among ELC and ILC populations in WT (red) and *Rag1*$^{-/-}$ (blue) models. Representative data from n>3.

The genetic ablation of *Rag* genes causes an intrinsic defect in T and B cell maturation in the context of otherwise normal immune anatomy. Contrastingly, the Nude mouse model is rendered effectively athymic by a mutation in the *Foxn1* gene that results in defective development of the thymic epithelium and the almost complete absence of T cells[265,266]. Interestingly, we analyzed epidermal cell suspensions of Nude mice and observed that, similarly to the *Rag1*$^{-/-}$, their epidermis also harbored a heterogeneous Thy1$^+$ population with a fraction of CD2$^+$ cells and ICOS$^+$ (Figure 8a and b). Consequently, we can possibly conclude that our population is thymic independent.

Given the thymic-independence of ELC generation, we asked which other components of the immune differentiation pathways these cells were reliant upon. The common gamma chain (γ_c), also known as IL-2R gamma (IL2Rγ), is a cytokine receptor subunit that is common to the receptor complexes for at least six different interleukin receptors: IL-2, IL-4, IL-7, IL-9, IL-15 receptors[267–272]. In consequence, γ_c-deficient mouse models, such as NOD *scid* gamma (NSG), lack all mature T cells, including DETCs, implicating that γ_c receptors do play an essential role in development of the DETC network in the skin[273]. NSG is considered the most resourceful immunodeficient mouse,

combining the features of the NOD/ShiLtJ background, the severe combined immune deficiency mutation (*scid*) and *Il2rg* deficiency. Hence, we tested if ELC were also γ_c-dependent. Examining the epidermis of NSG mice revealed a complete lack of $Thy1^+$ cells (Figure 8a and b). As a result ELCs were also completely absent in these animals, meaning that this population is dependent on the IL2Rγ for its survival or maintenance.

In addition, deletion of IL-15, IL-15Rα, or IL-2Rβ results in a block of NK cell development and impaired γδ T cell development in the epithelium[274–276]. Previous studies have shown that IL-15/IL-2Rβ plays an essential role in either maturation of $V\gamma3^+$ T cells in the fetal thymus or in expansion and/or survival of DETCs in the skin[277]. Therefore, we proceeded with the analysis of the epidermis of *Il15ra*$^{-/-}$ mice. As expected, these mice had no DETCs in their epidermis but their $CD3^-$ fraction could still be split into the same two populations as the other mouse models analyzed previously (Figure 8a). Curiously, ELCs were reduced in these mice (Figure 8b).

Thus, we conclude that ELCs are different from the skin ILC2 previously identified, are thymic independent but IL-2Rγ and IL-15Rα dependent.

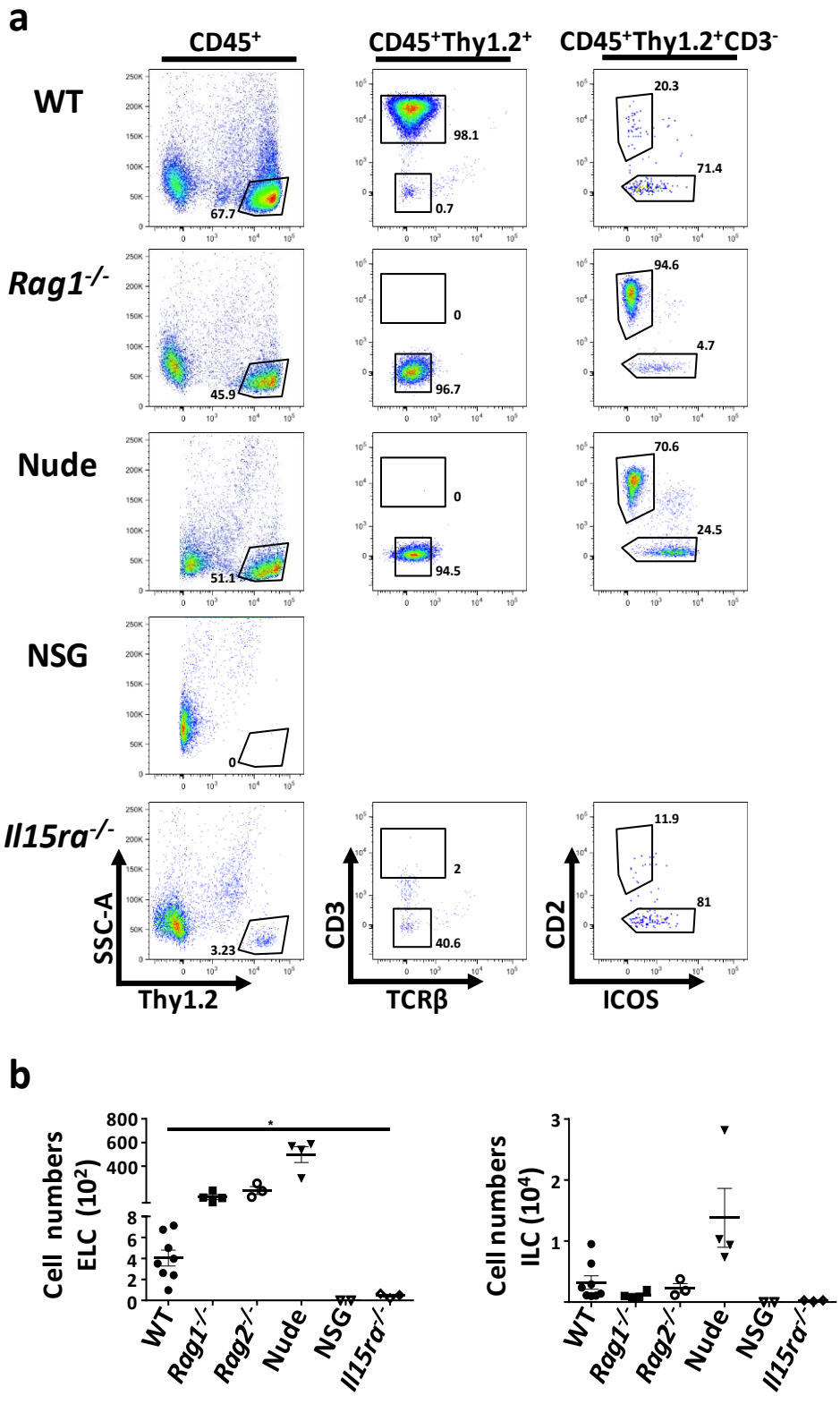


Figure 8. Presence of ELC in different animal models.

a. Flow cytometry of mouse epidermal cell suspensions of different 8wo mouse models. Representative gating strategies to identify ELC or ILC populations. **b.** Absolute numbers of ELCs and ILCs within the hematopoietic compartment in different animal models. Each data point represents an individual mouse. Representative data from n>3, except for NSG mice where n=2.

4.3 ELCs are radioresistant and possess self-renewal capacity

DETCs, similarly to Langerhans cells, are resistant to radiation. Both populations are derived from embryonic progenitors and renew themselves locally, not being replaced by any circulating/transplanted bone marrow progenitor[278,279].

Due to the unique characteristics of these epidermal subsets, we decided to test if ELCs share similar homeostasis cues with DETCs. By further characterization of the homeostasis of this population we hope to gain insight on their origin and nature.

Firstly, we generated bone marrow chimeras by reconstituting lethally irradiated *Rag1*$^{-/-}$ mice with WT bone marrow. Analyzing the epidermis 2 months after transplant revealed that, as expected, all the T cells found in the epidermis were from donor origin. Unsurprisingly 80% of the ILC population was also replaced by the donor cells (Figure 9), as ILC susceptibility to radiation has been described previously[50]. However, ELCs remained from the host 2 months after reconstitution (Figure 9). Similar observations were made at longer time point after transplantation (6 months), suggesting that the ELCs maintain themselves locally, independently of bone marrow input, in contrast to ILCs (Figure 9).

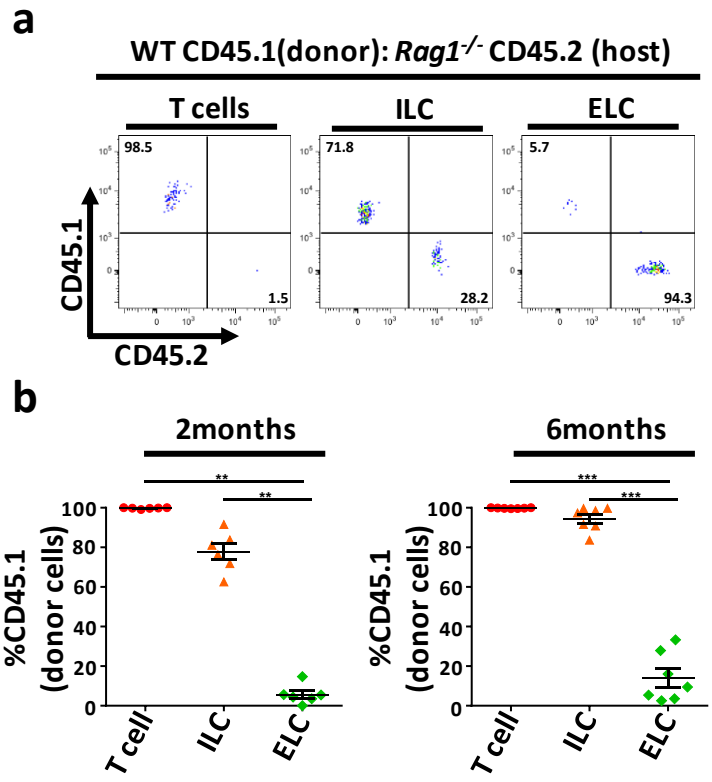


Figure 9. ELCs are radioresistant.

a-b. Flow cytometry data showing percentage of CD45.2 and CD45.1 T cells, ILCs and ELCs in mouse epidermal cell suspensions from BM chimeric mice (CD45.1 WT:CD45.2 *Rag1*$^{-/-}$) (2 months n=6; 6 months n=7). Each data point represents an individual mouse.

Bone marrow chimeras are often informative, but the process of irradiation and reconstitution results in an inflammatory environment that introduces experimental artifacts[280]. Therefore we also assessed the homeostatic turnover of epidermal cell populations using parabiotic mice. In this model, two adult congenic mice differing in expression of CD45 antigens, for example, are surgically attached in order to link their circulatory systems. This technique enables measurement of the extent of the contribution made by blood-borne cells from each parabiont to immune cell populations in the other, over prolonged periods and without the need for irradiation.

We joined congenic wildtype CD45.1 and *Rag1*$^{-/-}$ CD45.2 mice surgically and assessed the presence of non-host cells in the *Rag1*$^{-/-}$ four months later. As predicted all the T cells found in the *Rag1*$^{-/-}$ epidermis were from donor origin, while ILCs showed some heterogeneity in their ability to exchange between circulations. Surprisingly, 100% of the ELCs were kept from the host (Figure 10a-b).

To exclude the possibility that WT mice, which bear fewer ELCs than their *Rag1*$^{-/-}$ counterparts (Figure 5a), were simply unable to provide the appropriate blood-circulating precursor for ELCs, we confirmed our findings in *Rag1*$^{-/-}$ CD45.1 and *Rag1*$^{-/-}$ CD45.2 parabionts. Similarly, after four months, ELCs in the epidermis of both mice remained of host origin (Figure 10c-d). In summary, ELCs, similarly to DETCs and LCs, are resident cells that do not undergo replenishment from precursors arising from bone marrow or borne in the blood, either following irradiation or during prolonged periods of parabiosis.

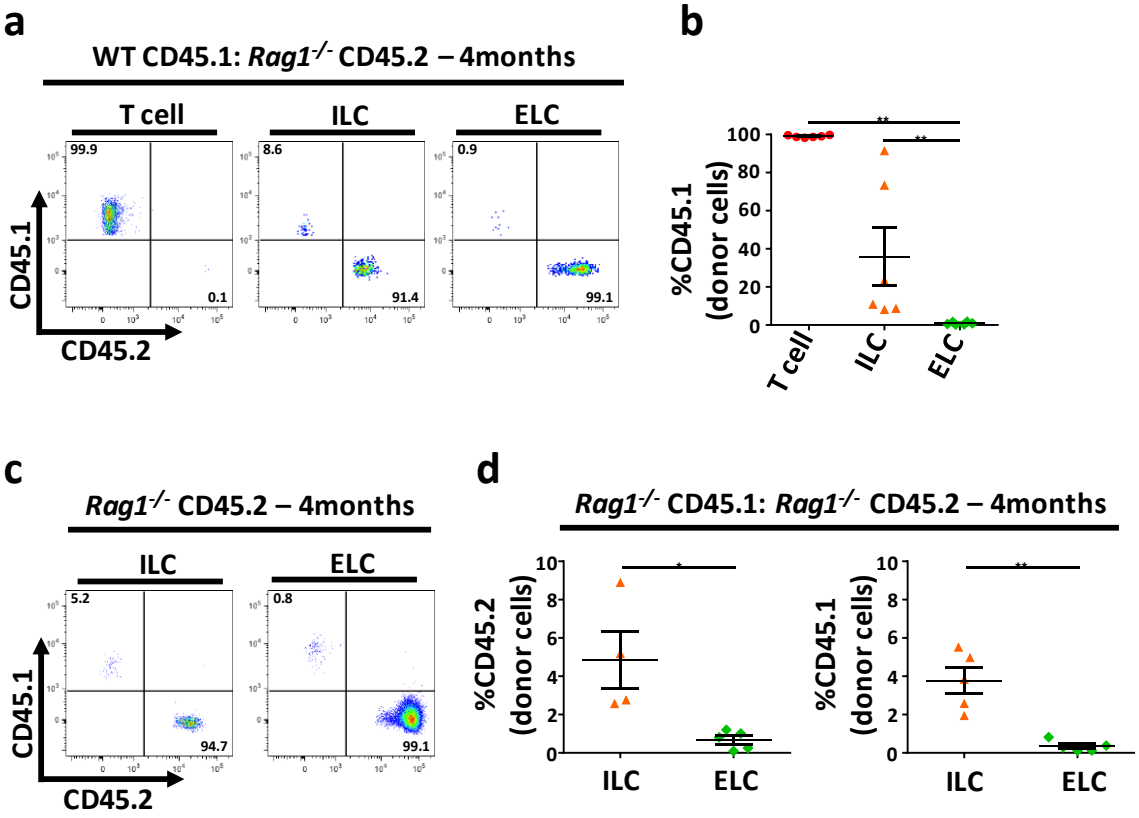


Figure 10. ELCs renew themselves locally.

a-b. CD45.1 WT mice were joined surgically with CD45.2 *Rag1*$^{-/-}$ mice to create parabiont pairs. Four months later flow cytometric analysis was performed on epidermal cell suspensions from the *Rag1*$^{-/-}$ mice, assessing the contribution of the donor to the cell pool. n=6. **c-d.** Parabiosis was performed with CD45.1 and CD45.2 *Rag1*$^{-/-}$ mice. Four months later epidermis from both mice was analyzed by flow cytometry. Representative plots of the donor contribution into the epidermal cell composition of each mouse. n=4. Each data point represents an individual mouse (a-d).

4.4 ELCs express heterogenous levels of T lineage markers

Since ELCs shared such unique characteristics with DETCs and expressed lymphoid markers we wondered if this population could belong to the T cell or T cell progenitor family.

Several studies have shown that T cell progenitors, in the adult or embryonic thymus, express CD3ε intracellularly[281,282] or that intracellular CD3 can be a hallmark of lymphoid cells belonging to the T cell lineage[283]. Therefore, we investigated whether this population expressed CD3 in their cytoplasm.

WT, *Rag1*$^{-/-}$ and Nude mice epidermal cell suspensions were analyzed by flow cytometry. Results were striking; while cytoplasmic expression of CD3ε in the ELC population varied between 25 to 40% in all models, ILCs did not express any intracellular CD3ε (Figure 11a-b).

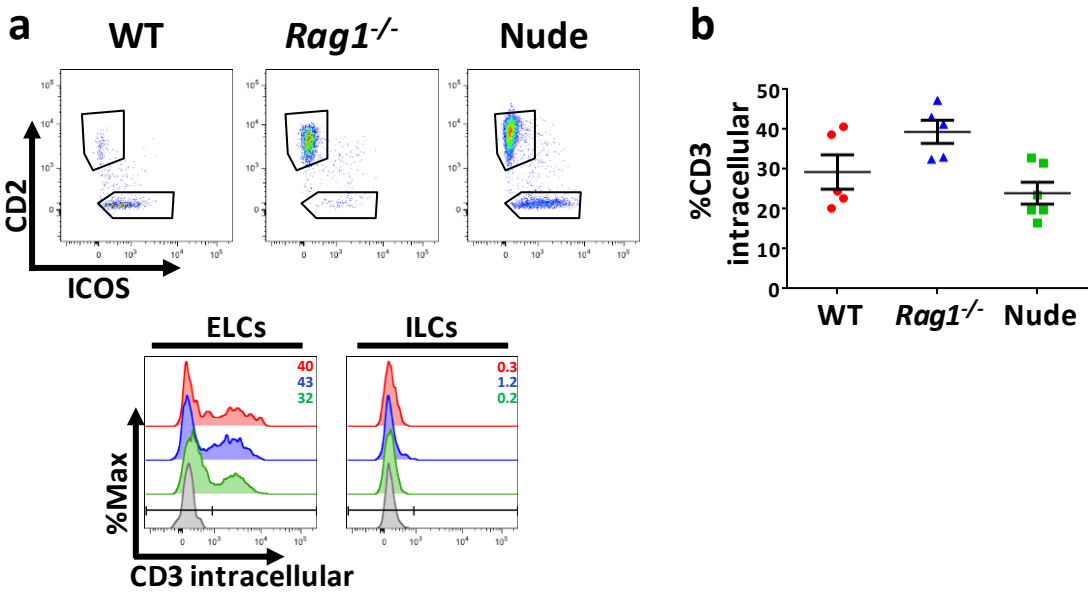


Figure 11. ELC population expresses CD3ε intracellularly.

a. Flow cytometry of WT, *Rag1*$^{-/-}$ and Nude epidermal cell suspensions with intracellular labeling for CD3ε. Histograms represent the percentage of cells that express CD3ε in their cytoplasm in the ELC and ILC populations. Representative flow cytometry plots from n=5. **b.** Dot plot showing the expression of CD3ε intracellularly within the ELC population in WT (red), *Rag1*$^{-/-}$(blue) and Nude (green) mouse models. Each data point represents an individual mouse, n=5.

Another phenotypic characteristic that links a cell's nature to the T cell lineage is the expression of pre-TCRα (pTα). The pTα chain is an essential and invariant subunit of the pre-TCR[284]. The only known physiological function of pTα protein is to associate with nascent TCRβ chains in committed T lineage progenitors. Together they form a functional pre-TCR, which provides essential signals to promote development of αβ thymocytes and to regulate αβ/γδ lineage choice. In line with this highly restricted function, pTα expression is largely confined to immature thymocytes. On top of that, another study has shown that pTα expression is found in pro-T cells outside the thymus as well as in intra- and extra-thymic sites of T cell development[285].

Therefore, we took advantage of a CRE based fate mapping system of pTα, where all cells that have expressed pTα are heritably marked [248]. To visualize iCre expression, *Ptcra*^{iCre} knockin mice were intercrossed with *Rosa26*^{tdRFP} reporter line[286] (Figure 12a-b). As a negative control *Rosa26*$^{tdRFP/tdRFP}$ mice lacking iCre expression were used. By analyzing 8wo mice, we observed that, as expected, all αβ T cells expressed pTα. However DETCs also express nearly 100% (Figure 12). It is controversial whether γδ T cells can express pTα, and even though some evidence of that has been published[287–289], the precise number of γδ T cells which pass through a pTα-expressing developmental stage, under physiological *in vivo* conditions, has not been determined yet. Under our experimental conditions, we can conclude that all DETCs pass by a pTα-expressing developmental stage. Intriguingly, ELCs also partially expressed pTα, around 12%. ILCs also exhibited a small expression of pTα, around 4% (Figure 12).

We also analyze the same fate mapping model in a *Rag1*$^{-/-}$ background. However, ELCs had no pTα expression in this model. We hypothesize that the lack of the *Rag1* expression may limit the ability for ELCs to proceed in the normal development of T cells. Possibly the *Rag* expression is upstream of the *Ptrca*, leading to limitation of the expression of this protein in these cells.

Thus, partial expression of CD3ε intracellular and pTα led us to presume that ELCs are a pool of T-lineage committed cells that, for unknown circumstances, are incapable of continuing down the T cell development pathway.

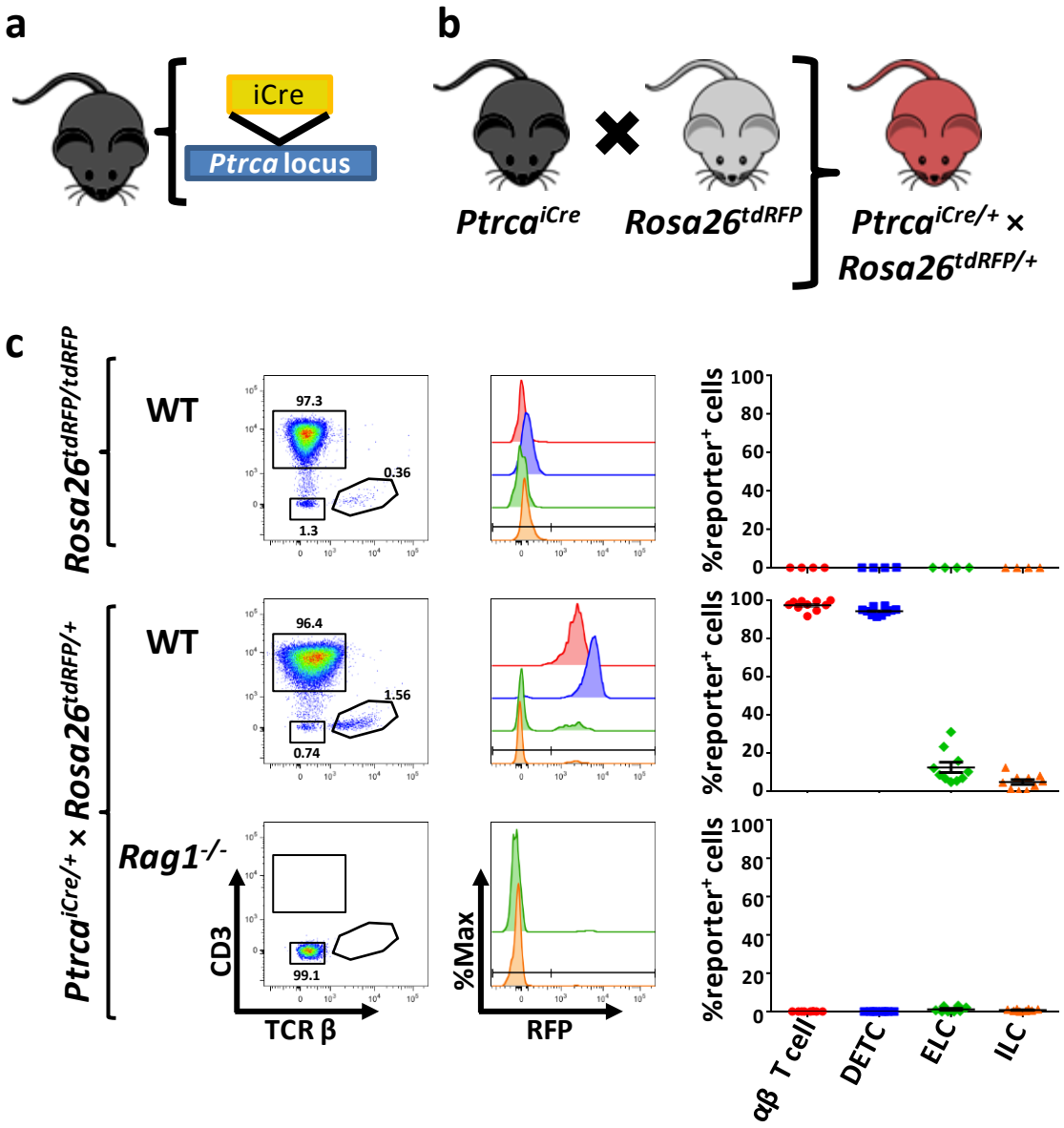


Figure 12. ELC population patially expresses pTα.

a. Strategy for the pTα^{iCre} Knockin mice construction. **b.** Strategy to create *Ptrca*$^{iCre/+}$ x *Rosa26*$^{tdRFP/+}$ mice. **c.** Representative flow cytometry plots to identify pTα-expressing cells in the mouse epidermis. The histograms and the dot plots show the expression of pTα within the αβ T cell (red), DETC (blue), as well as ELC (green) and ILC (orange) populations. Each data point represents an individual mouse. Data were obtained from n=4 of *Rosa26*$^{tdRFP/tdRFP}$ control mice, *Ptrca*$^{iCre/+}$ x *Rosa26*$^{tdRFP/+}$ n=10 on WT background and n=8 on *Rag1*$^{-/-}$ background.

4.5 Adult ELCs do not differentiate into T cells

The fate mapping data showed that some of the ELCs from WT mice had differentiated sufficiently far down the T cell pathway to express pTα. However, this was not the case in *Rag1*$^{-/-}$ mice, perhaps because the lack of *Rag* expression created an intrinsic block at a stage preceding pTα expression.

In an attempt to rescue ELC development in *Rag1*$^{-/-}$ mice, we developed a *Rag*-inducible model (Figure S2, *see material and methods section*). A DNA fragment containing 2A peptide sequences between *Rag1* and *Rag2* cDNA after the loxP flanked Stop cassette was inserted into the *Rosa26* locus of WT Embryonic Stem (ES) cells. The resulting mouse model was crossed to *Rag1*$^{-/-}$ Rosa26$^{\text{Cre-ERT2/+}}$ [290], resulting in *Rag1*$^{+/-}$ mice harboring an inducible *Cre-ERT2* transgene or the *stop-Rag1A2* fragment in their *Rosa26* locus, respectively. Mice were then further intercrossed to obtain the desired genotype, the *Rag*-inducible model on *Rag1*$^{-/-}$ background (Figure 13).

Upon tamoxifen administration that activates the Cre recombinase, these mice produce both Rag1 and Rag2 protein from *Rosa26*^{Rag1A2} allele as well as express GFP as a reporter of recombination, allowing identification of *Rag*-expressing cells by flow cytometry (Figure 13).

We hypothesized that by inducing expression of the *Rag* genes that the cells were missing, the developmental potential of ELC would be restored, with the possibility of proceeding from their double negative (DN) CD4^{-}CD8^{-} status to the DP[291], or subsequent single positive CD4 (CD4^{+}CD8^{-}, SP CD4^{+}) or CD8 (CD4^{-}CD8^{+}, SP CD8^{+}) stages[292].

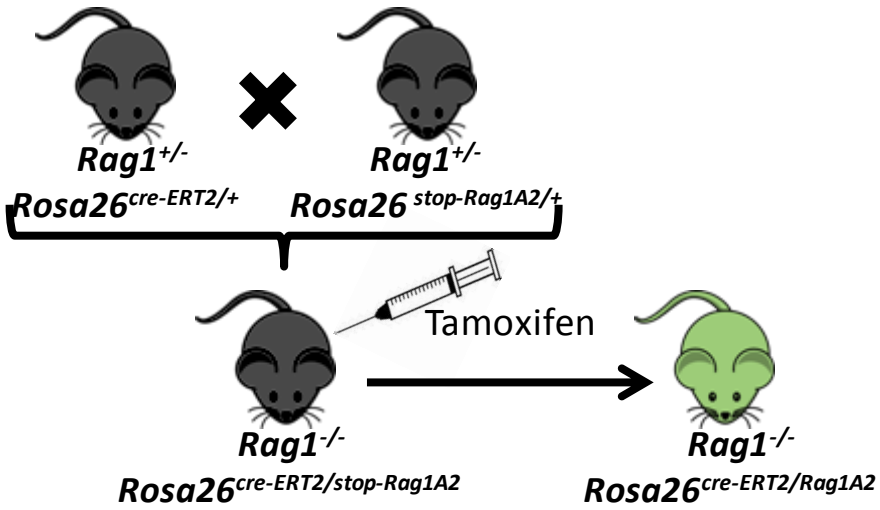


Figure 13. *Rag*-inducible model strategy.

Rag1$^{-/-}$ mice were crossed with WT mice either with a *cre-ERT2* insertion or a *stop-Rag1A2* insertion into their *Rosa26* locus. The resulting mouse models were crossed, giving rise to the *Rag*-inducible model (*Rag1*$^{-/-}$*Rosa26*$^{cre\text{-}ERT2/stop\text{-}Rag1A2}$). Upon tamoxifen administration the Cre recombinase becomes active leading to the re-expression of the *Rag* genes that can be identified by flow cytometry by the expression of the GFP.

Rag gene expression in our inducible model was induced by intraperitoneal (i.p.) administration of Hydroxytamoxifen (4'OHT) for 5 consecutive days. On month later, both thymus and epidermis of WT, *Rag1*$^{-/-}$ and the *Rag*-inducible mice were collected and their cell populations analyzed by flow cytometry.

By analysis of the WT thymocytes, we could observe a normal T cell development, where TCRβ expression was restricted to SP stage, a sign of T cell maturation (Figure 14). In the *Rag1*$^{-/-}$, due to the absence of the *Rag1* gene, thymocytes were, as expected, arrested at the DN stage (Figure 14). Approximately one third of thymocytes in the *Rag*-inducible model expressed GFP, and 12% of these thymocytes had proceeded to the DP stage, while all thymocytes in the *Rag1*$^{-/-}$ were arrested at the DN stage (Figure 14). Furthermore, in the *Rag*-inducible mice, 0.5-1% of thymocytes achieved SP expression of CD4 or CD8 with TCRβ expression (Figure 14), showing that our rescue model was able to partially overcome the blockade of thymic T cell development seen in *Rag1*$^{-/-}$ mice.

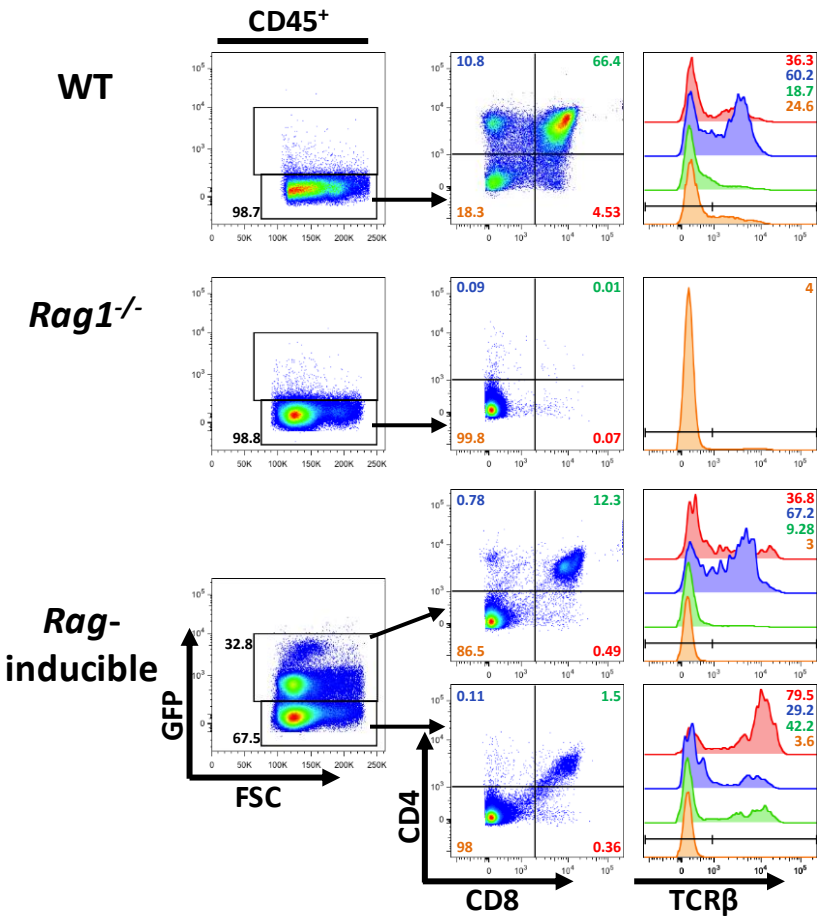


Figure 14. *Rag*-inducible model can rescue T cells in the thymus.

Mice were injected i.p. with tamoxifen daily for 5 days and thymocytes were analyzed 30 days later by flow cytometry. GFP signal indicated *Rag* recombination and expression. Representative flow cytometry plots of thymocytes of WT and *Rag1*$^{-/-}$ controls, as well as the *Rag*-inducible model are shown. Data representative of 3 independent experiments with 2 mice each.

Next we analyzed the epidermis of the same mice. While selecting the Thy^{hi}CD2^{+} cells in the WT we have included DETCs. Therefore, the results were unsurprising; these cells were CD4^{-}CD8^{-}TCRβ^{-} but TCRγ^{+}CD3^{+} (Figure 15). In the *Rag1*$^{-/-}$ these cells were negative for all the markers analyzed (Figure 15). However, in the *Rag*-inducible model ELCs exhibited comparable phenotype regardless of the induction of *Rag* expression (Figure 15): even upon reinstatement of the *Rag* genes, no change in expression of markers linked to T cell maturation, such as TCR or CD3, was observed (Figure 15). Later time points and topical delivery of 4'OHT revealed similar results (*data not shown*).

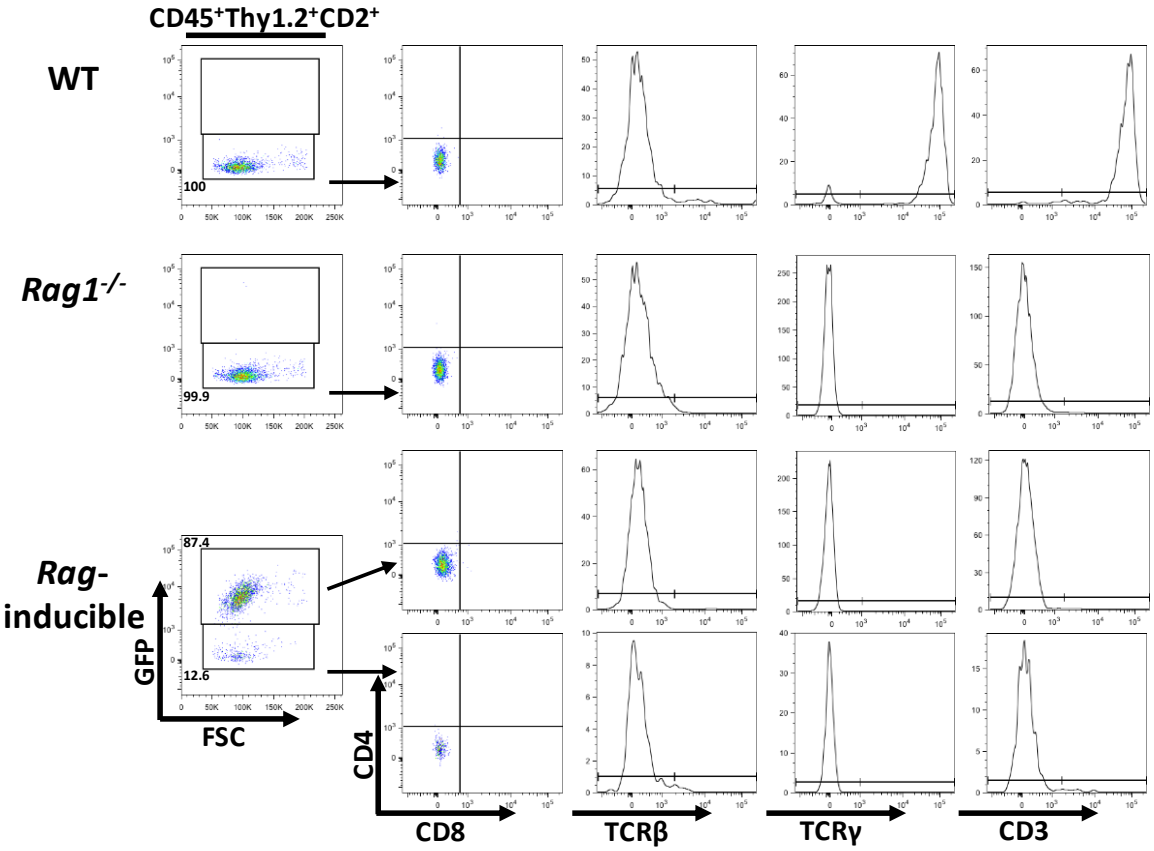


Figure 15. *Rag*-inducible model fails to rescue T cells in the epidermis.

Mice were injected i.p. with tamoxifen daily for 5 days and epidermal cell suspensions were analyzed 30 days later by flow cytometry. GFP signal indicated *Rag* recombination and expression. Representative flow cytometry plots of WT and *Rag1*$^{-/-}$ controls, as well as the *Rag*-inducible model are shown. Data representative of 3 independent experiments with 2 mice each.

Hence, we believe that either ELCs are not T cell progenitors or the adult skin environment is not the most appropriate to proceed with the development of T cell progenitors.

4.6 ELCs are present in the murine epidermis prior to birth

DETCs arise from embryonic precursors and self-maintain into adulthood [278,293,294]. Given the homeostatic similarities we uncovered between ELCs and these cells, we asked whether ELCs might also seed the skin prior to birth. To pursue this new hypothesis we analyzed the epidermis of both WT and *Rag1*$^{-/-}$ before and right after birth, at E17.5 of embryonic life, newborn (NB) and adulthood (Figure 16a-b).

Within the CD45^{+}Thy1^{+} pool of cells the presence of T cells was assessed through the expression of CD3. To confirm that the CD3^{+} cells found in the WT epidermis at E17.5 were DETCs, we verified the expression of their specific γδ TCR, Vγ3. As has been reported in the literature[294], DETCs appear in the epidermis as early as E17.5 and reside there throughout development. Curiously, at the same timepoint, a CD3^{-}Vγ3^{-} population was also found in the WT and *Rag1*$^{-/-}$ epidermis (Figure 16a). Throughout development, the WT CD3^{-}Vγ3^{-} population slightly increases its frequency at birth but rapidly decreases during adulthood. In the *Rag1*$^{-/-}$ epidermis these cells followed a similar pattern to the one observed by DETCs in the WT epidermis, increasing its frequency throughout development (Figure 16b).

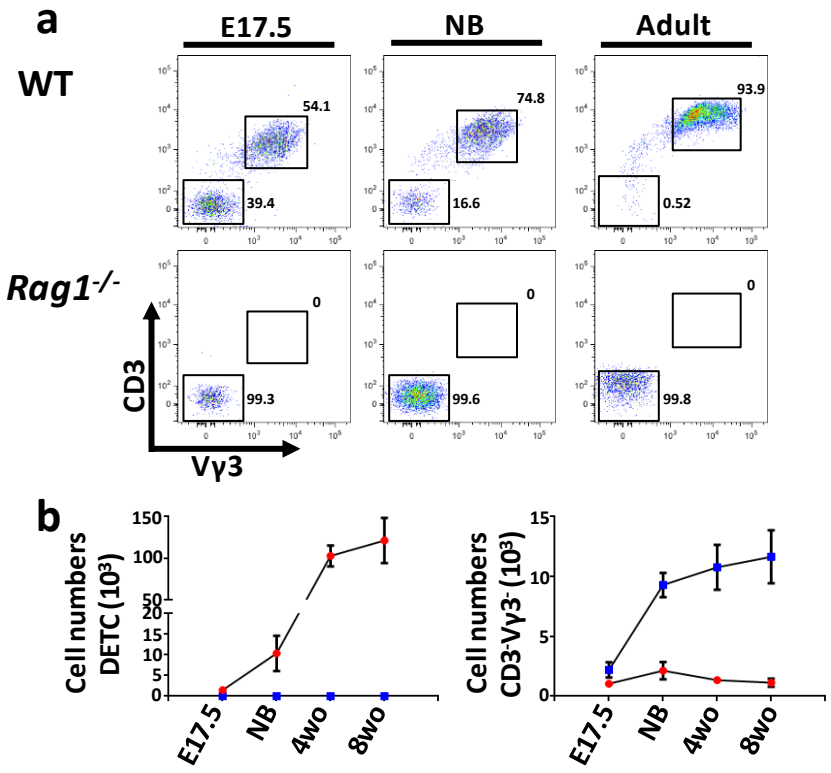


Figure 16. Thy1^{+}CD3^{-} population is present before birth in both WT and *Rag1*$^{-/-}$ models.

a. Flow cytometry of mouse epidermal cell suspensions. Gating strategy of DETC and Thy1^{+}CD3^{-} populations are shown throughout mouse development. **b.** Absolute numbers of both populations during late embryogenesis, newborn and adult mice are shown for WT (red) and *Rag1*$^{-/-}$ (blue). Representative data from n>3.

To further characterize this CD3^{-}Vγ3^{-} population and to understand if the heterogeneity observed in the adult was the same earlier in life, we explored the expression of CD2 and ICOS. Results were striking as hardly any ILCs were present before birth, showing that 70% of the CD3^{-}Vγ3^{-} cells were positive for CD2 in the WT epidermis, corresponding to the ELCs previously observed in the adult epidermis. The same was observed in the *Rag1*$^{-/-}$ epidermis before birth (Figure 17a). At the newborn (NB) stage the frequency of ELCs increased, further expanding until six days after birth (D6) in both models. However, by adulthood, ELC frequency had decreased, reaching almost undetectable numbers in the WT (Figure 17b). ILCs are only properly detected after birth, expanding until adulthood (Figure 17b).

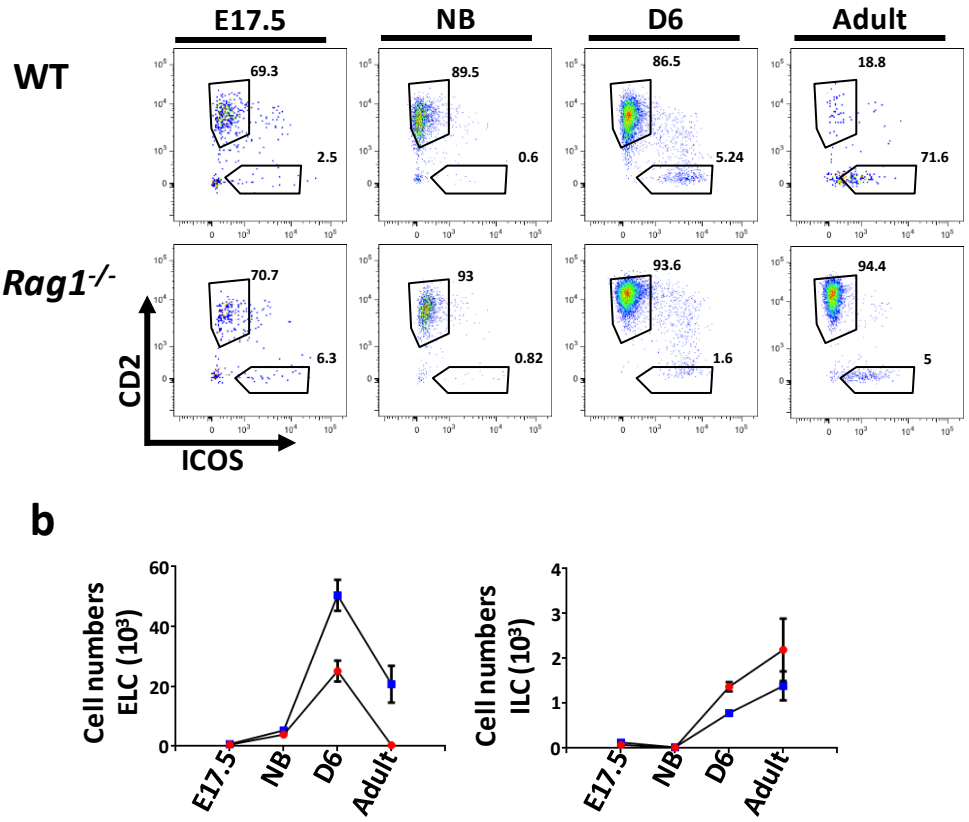


Figure 17. ELC population is present before birth in both WT and *Rag1*$^{-/-}$ models.

a. Dot plots show the gating strategy for the expression of CD2 and ICOS within the CD3^{-}Vγ3^{-} population in the mouse epidermis of both models, WT and *Rag1*$^{-/-}$. **b.** Absolute numbers of both populations in late embryogenesis (E17.5), newborn, day6 and adult mice for WT (red) and *Rag1*$^{-/-}$ (blue). Representative data from n>3.

In addition, we screened different tissues of WT NB mice in order to understand if this population was restricted to the epidermis. This hematopoietic population of Thy^{hi}CD3^{-}αβ/γδTCR^{-}CD2^{+} cells was detected in tissues where T cell progenitors are abundant such as the thymus. However, the epidermis stood out as presenting a high ratio of ELCs among the pool of CD45^{+} cells in the different tissues (Figure S3).

Altogether we can conclude that in late embryogenesis and after birth, WT mice have a population of ELCs in similar frequencies as the *Rag1*$^{-/-}$ mice. These findings are in concordance with a theory that ELCs and DETCs might be competing for the same niche, leading to the almost disappearance of ELCs upon the high proliferation of DETCs throughout development.

4.7 Embryonic ELCs do not express extracellular CD3ε but express pTα

To understand if, similarly to the ELCs found in the adult, the embryonic population of ELCs was bound to the T cell lineage, we analyzed late embryogenesis time points for the CD3ε expression in their cytoplasm as well as the pTα fate mapping model previously used.

Epidermis from WT embryos at E17.5 was analyzed by flow cytometry for the presence of CD3ε intracellular in the DETC and ELC populations. As expected, DETCs had close to 100% expression of CD3ε in their cytoplasm (Figure 18a-b). However, at this stage, the presence of intracellular CD3ε in the ELC population was barely detectable (Figure 18a-b). We hypothesize that before birth it is too early for these progenitors to start expressing CD3ε in their cytoplasm. Nevertheless, NB WT mice were also analyzed exhibiting the same results (*data not shown*) dismissing such hypothesis.

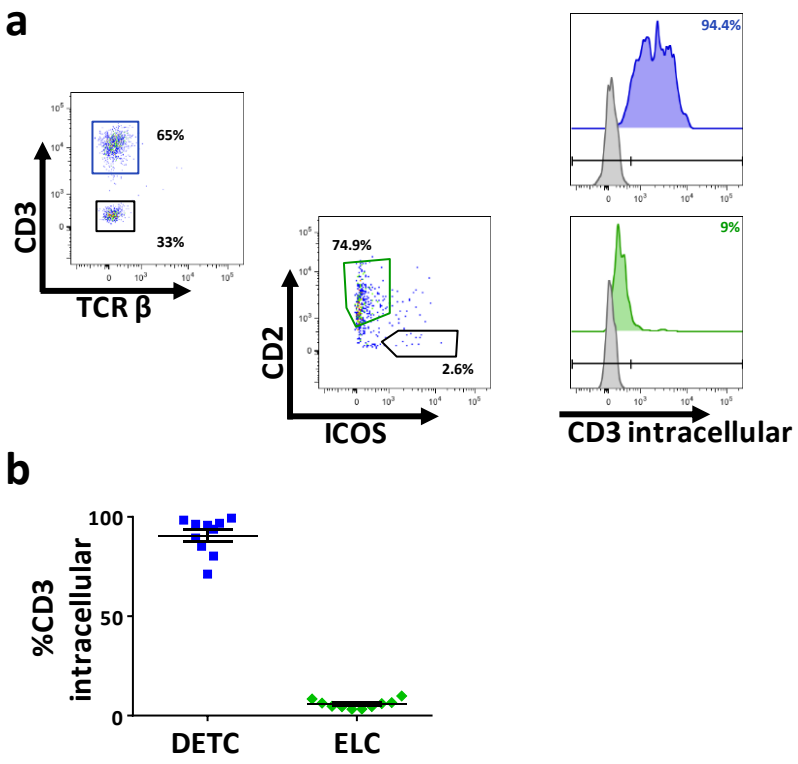


Figure 18. Embryonic ELCs do not express CD3ε in their cytoplasm.

a-b. Flow cytometry of mouse epidermal cell suspensions with intracellular labeling for CD3ε of E17.5 WT embryos. Histograms and dot plot identify the percentage of CD3ε intracellular expression of both DETC (blue) and ELC (green) populations. Each dot represents one individual embryo. Representative data from 10 embryos from 3 independent experiments.

Even though ELCs did not express CD3ε in their cytoplasm we wondered if they would express pTα before birth, similar to the adult population. We proceeded with flow cytometry analysis of the epidermis of E18.5 embryos of the pTα fate mapping model used previously. Our results showed that already at this time point DETCs were nearly 100% positive for pTα. However ELCs showed to be heterogeneous for pTα expression, with an average of 35% expression (Figure 19a-b).

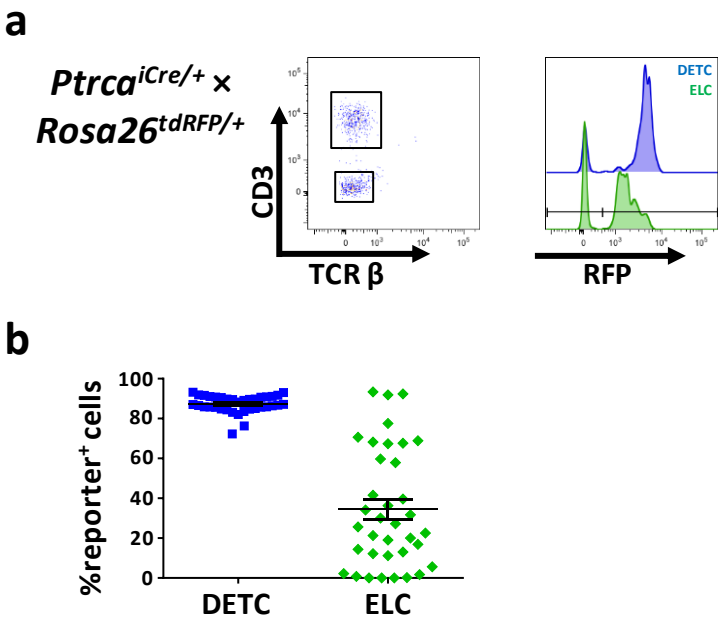


Figure 19. Embryonic ELCs express pTα.

a-b. Gating scheme to identify pTα expressing cells in the E18.5 mouse epidermis. The histograms and dot plot identify pTα-expressing cells within the DETC (blue) as well as ELC (green) populations. Each data point represents an individual embryo. Data were obtained from n=36 embryos from 6 independent experiments of *Ptrca*$^{iCre/+}$ × *Rosa26*$^{tdRFP/+}$.

Thus, we conclude that embryonic ELCs in the WT seem to belong to the T cell lineage due to the fact that these cells do express pTα. The lack of CD3ε expression in their cytoplasm could be associated with a very immature phenotype.

4.8 Neonatal ELCs do not differentiate into T cells

Since neither adult nor embryonic skins seem to provide the correct environment for the ELC population to further develop and to reveal a potential intrinsic loss during development in the skin, we decided to address this question through *in vitro* and *ex vivo* experimental designs.

Purified ELC population from WT NB epidermis was co-cultured with TSt-4 stromal cells expressing Delta-like 1 (TSt4/DL1). The fibroblastoid cell line TSt-4, established from fetal thymus tissue of C57BL/6, expressing delta-like 1 (DL1), has shown to support the development of T cell progenitors into mature T cells[295]. Cytokines such as IL-2, IL-7 and IL-15 were added to the culture in order to promote the development of these T cell progenitors. After 9 to 12 days in culture the ELC population maintained its phenotype, lacking any maturation markers of the T cell lineage, such as CD3 or TCR (Figure 20).

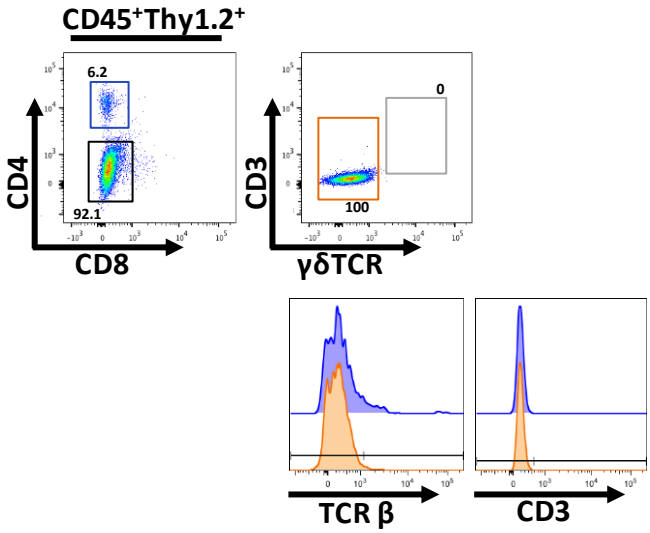


Figure 20. ELCs co-cultured with TSt4/DL1 stromal cell line.

Progeny of ELCs co-cultured for 9-12 days with TSt-4/DL1 stromal cells were analyzed by flow cytometry. Expression levels of CD4, CD8 and mature T cell markers such as TCR and CD3 were analyzed. Data representative of n=3.

Fetal thymic organ cultures (FTOC) have been used for over 25 years to address scientific questions regarding the mechanisms of T cell development. For example, FTOC systems have been used to access the potential of T cell progenitors from the fetal

liver[296]. Therefore, these culture systems are considered the most accurate way to address the nature of a T cell progenitor *ex vivo*.

We cultured E15.5 thymic lobes from CD45.1 WT mice with 2'-deoxyguanosine (dGuo) for 7 days. Treatment with dGuo selectively eliminates both thymocytes and interdigitating cells/dendritic cells (IDC/DC) from the thymic cultured tissue[297]. Purified NB ELC, and thymic DN populations as positive control (Figure S4), were added to the hematopoietic-depleted-lobes in culture by the hanging-drop system where they were cultured for 12 to 15 days.

When added to the FTOC system, thymic DN developed like they would do *in vivo*. DP and both SP can be detected with upregulation of TCRβ and CD3 (Figure 21a). Some γδ T cells could also be detected. In contrast, ELCs in FTOC maintained their Thy^{hi} profile, down-regulated CD2 expression, and did not upregulate any TCR, CD3 or CD8 (Figure 21b). Even though approximately 5% of cultured ELCs did express CD4 as their *in vivo* counterpart (*data not shown*), they did not exhibit any other aspects of late DN, DP or SP thymocyte phenotype (Figure 21b).

Upon these results we can then conclude that neonatal ELCs are not T cell progenitors.

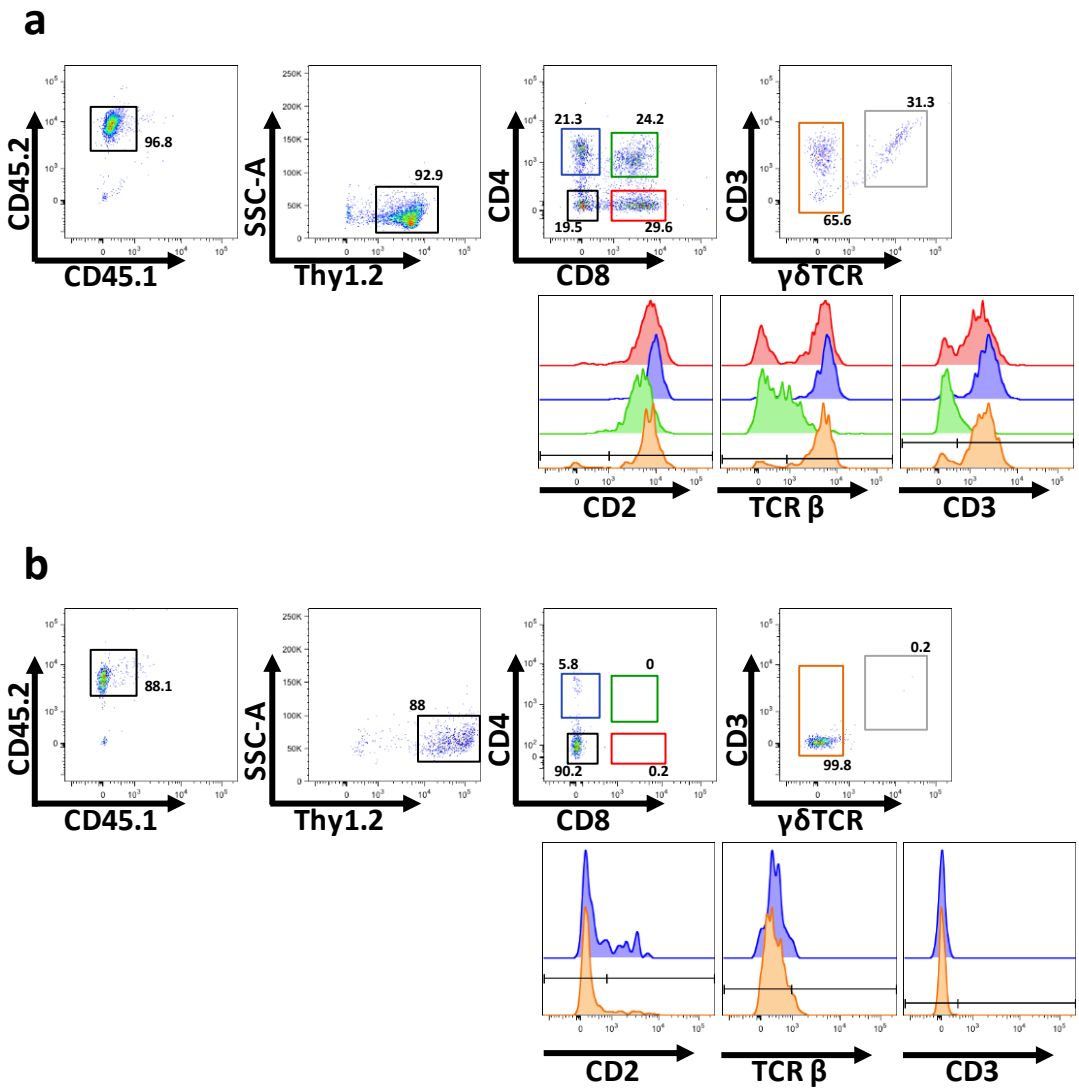


Figure 21. ELCs into FTOC system.

a-b. Progeny of the thymic DN (a) and ELCs (b) in fetal thymic organ culture for 12 to 15 days were analyzed by flow cytometry. T cell lineage markers such as CD4, CD8 and CD2 were used in this analysis, as well as maturation markers such as TCRβ, γδTCR and CD3. Data representative of n=5.

4.9 Neonatal ELCs express Natural Killer markers

As described before, CD2 and Thy1 are not restricted to the T cell lineage, since they can also be found in the surface of NK cells. Therefore, and since ELCs failed to further develop into T cells in *in vitro* and *ex vivo* culture systems, we hypothesized that the ELC population might belong to the NK-like family.

Murine NK cells are characterized by specific markers on their surface such as NK1.1 and CD49b. Therefore, we phenotyped the ELC population for these markers, as well as others linked to the NK family. Both WT and *Rag1*$^{-/-}$ ELC populations expressed low levels of CD49b and had a partial expression of NK1.1 (Figure 22a and b).

Interleukin 2 (IL-2) is a major growth factor for mature NK cells[298,299]. Freshly isolated NK cells preferentially express IL-2Rβ, through which IL-2 plays a pivotal role in proliferation and induction of cytolytic activity[300]. Thus, we investigated the expression of IL-2Rβ in the NB populations of WT and *Rag1*$^{-/-}$ animal models and found that both expressed low levels of such receptor (Figure 22a and b).

The CD2/SLAM family includes several members such as CD2, 2B4 (CD244) and CD48. Murine CD244 is expressed on different subsets of cells, such as T cells, mast cells, αβ T cells, and all NK cells[301–303]. In addition, CD48 is expressed widely on hematopoietic cells including T and NK cells and has been identified as a ligand for CD244 and CD2[304]. By analyzing the expression of both CD244 and CD48, we found that ELCs in both models expressed high levels of these molecules (Figure 22a and b). Of note that CD244 and CD48 expression was been reported to be expressed by DETCs[302] and LCs[305] respectively. While analyzing the NB epidermis we also observed that either DETC or LCs populations expressed both CD244 and CD48 (Figure S5).

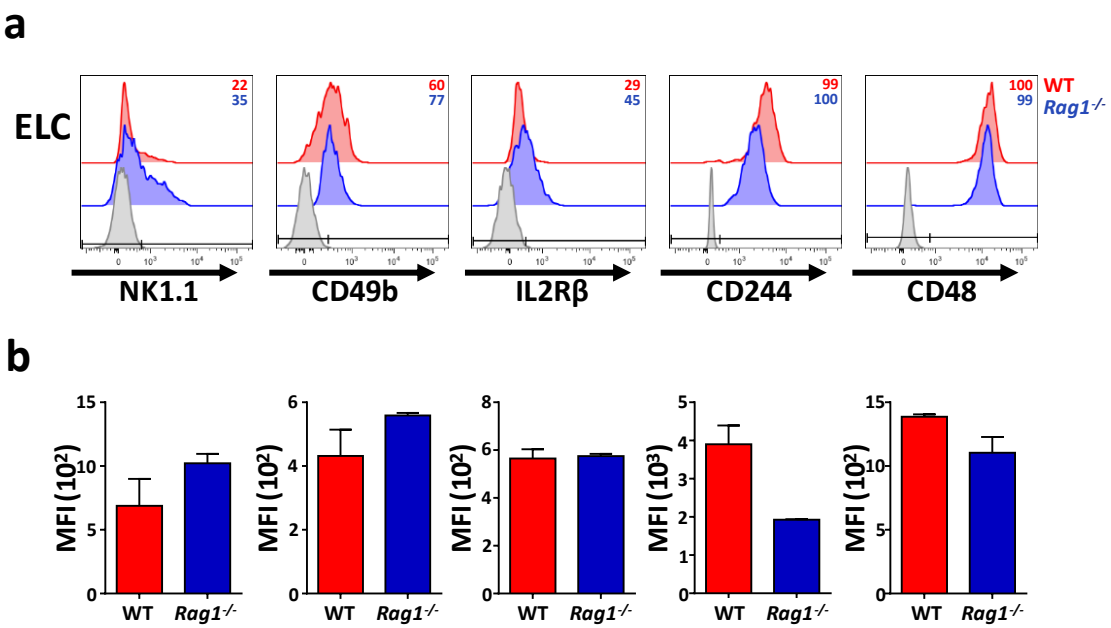


Figure 22. ELCs express NK markers.

a. Representative histograms of the level of expression of NK-like markers in the ELC population from epidermal cell suspensions of NB from both WT and *Rag1*$^{-/-}$ models. **b.** Mean Fluorescence Intensity (MFI) representation with standard deviation (SD) bars of the intensity of the NK-like markers in the ELC population from both WT and *Rag1*$^{-/-}$ mice. Data representative n>3**.**

4.10 Neonatal ELCs produce IFN-γ and IL-2

In order to search for other molecular cues that relate ELCs to the NK lineage we purified this population from WT NB mice and proceeded with NanoString analysis. NanoString is a multiplexed target profiling of up to 800 transcripts in a single reaction. In this experimental design, cells were analyzed for their level of expression for 179 genes known to be differentially expressed during inflammation (Table 1). These represent a broad range of relevant pathways such as apoptosis, interleukin signalling, Toll-like receptor signalling and others.

NanoString data was split into different categories according to their function. Expression of transmembrane receptors, transcription regulators, growth factors and G-protein coupled receptors are depicted in Figure S6, while expression of kinases, enzymes, and others are represented in Figure S7. Interestingly, the ELC population exhibited high expression for IL-18Rα, another molecule that is highly related to the NK lineage. Priming of NK cells by IL-18 is crucial for these cells to display full effector activity[306]. In addition, the transcription factors Fos and Jun revealed to be highly expressed by neonatal ELCs. Notably, in NK cells, IL-2 differentially regulates the expression of several transcription factors, including Jun and Fos[307].

In addition, we decided to validate at the protein level their cytokine profile since the NanoString data revealed that neonatal ELCs had high expression of IFN-γ, IL-13, IL-2 and TNF-α (Figure 23a). Hence, we proceeded with intracellular labeling of these cytokines in the ELC population under non-stimulated and stimulated conditions with PMA and ionomycin (Figure 23a and d). Under unstimulated conditions, ELCs from both WT and *Rag1*$^{-/-}$ NB expressed hardly any cytokine (Figure 23b and d). In contrast, upon stimulation ELCs from both mouse models expressed around 20% of IL-2 and IFN-γ, yet no IL-13 or TNF-α was detected (Figure 23c and e). IL-2 and IFN-γ production corroborates the hypothesis of these cells being part of an NK-like population.

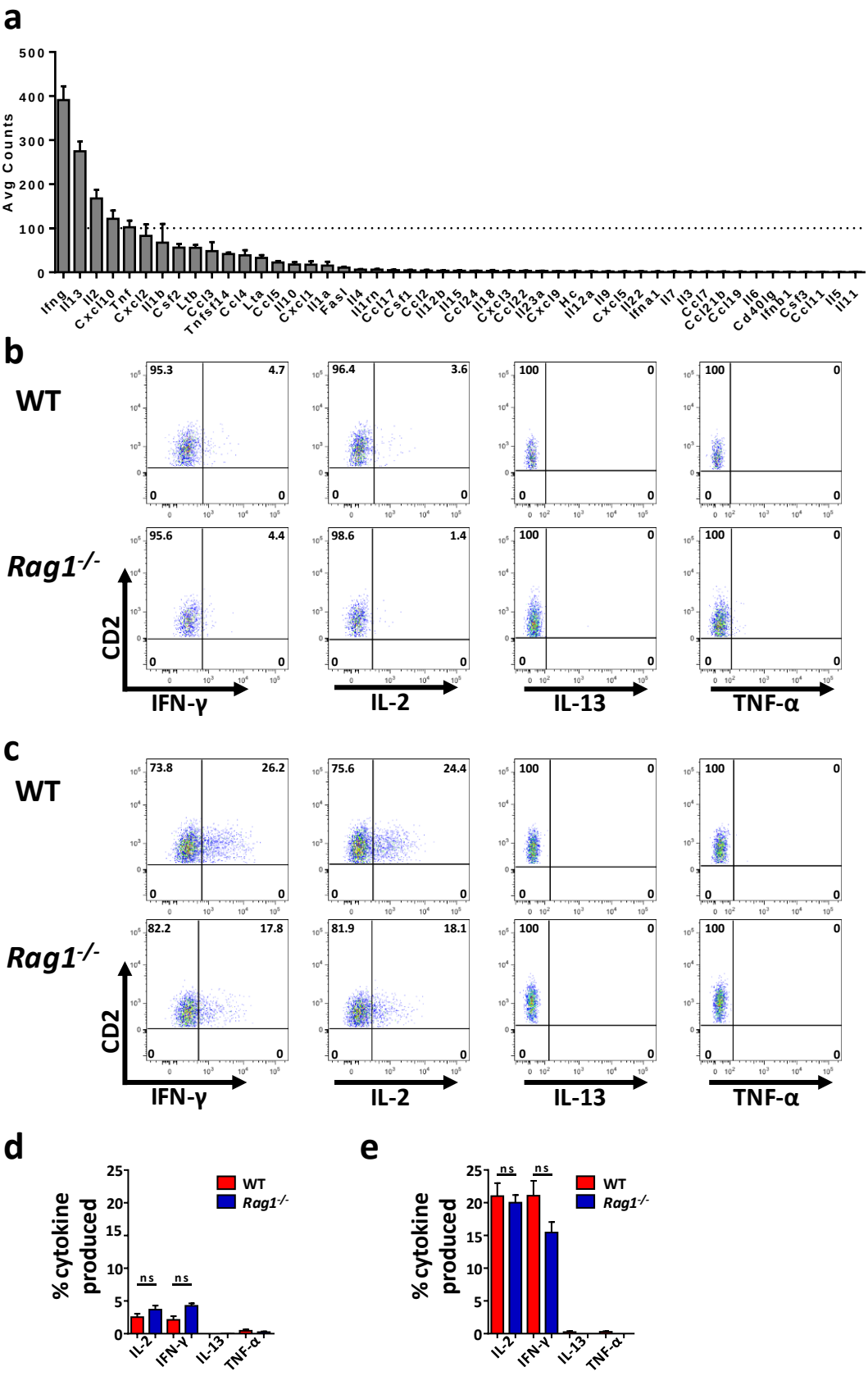


Figure 23. Cytokine production profile of neonatal ELCs.

a. Cytokine profile of expression by NanoString analysis of purified ELCs from NB WT. **b-e.** Representative dot plots and bar graphs of the production of IFN-γ, IL-2, IL-13 and TNF-α from ELCs from WT and *Rag1*$^{-/-}$ NB under unstimulated (b and d) and stimulated (c and e) conditions. Data representative of n>3.

4.11 Neonatal ELCs are Runx3 and Nfil3 dependent

Another possible approach to clarify the nature of this population is by their transcription factor dependency profile. Unfortunately, the NanoString cartridge selected did not have the typical NK-related transcription factors. So in order to further investigate this, we took advantage of different deficient mouse models for specific transcription factors.

The transcription factor Runx3 is known to be expressed in NK cells, and recently has been shown to regulate their activation through IL-15[308]. Since *Runx3*$^{-/-}$ mice have an embryonic lethal phenotype, dying at E19 to E20, therefore we did the analysis at the E17.5 stage. Embryonic ELCs are dependent on the transcription factor Runx3 since deficient embryos had a significant reduction in their population when compared to the WT at E17.5 (Figure 24a-b).

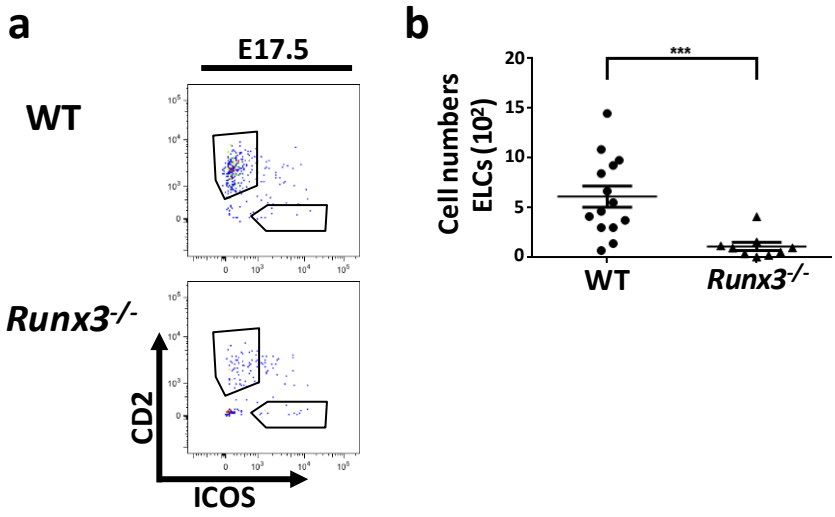


Figure 24. ELCs are dependent on Runx3.

a. Representative dot plots of ELC population in both WT and *Runx3*$^{-/-}$ E17.5 embryos analyzed by flow cytometry are shown. **b.** Dot plot identifies the absolute numbers between both mouse models. Each data point represents an individual embryo. Data were obtained from n=14 (WT) and n=9 (*Runx3*$^{-/-}$) embryos from 3 independent experiments.

In addition to the *Runx3*-deficient model, newborn epidermis from other deficient mouse models such as *Rorc*$^{-/-}$, *Tbx21*$^{-/-}$ and *Nfil3*$^{-/-}$ mice were analyzed by flow cytometry.

RORγt (RAR-related orphan receptor gamma, encoded by *Rorc*) has been highly involved in the development of innate lymphoid populations, more specifically ILC3[175]. When analyzing the ELC population in NB from *Rorc*$^{-/-}$ we noticed that this population is slightly expanded in this model when compared to the WT (Figure 25a-b).

Nfil3 (Nuclear factor interleukin-3, also known as E4-binding protein 4 (E4BP4)) has shown to be required for the development of all innate lymphoid cell subsets[156]. In particular for NK development, Nfil3 has shown to be crucial at driving their development and maturation *in vivo*[309,310]. Therefore *Nfil3*$^{-/-}$ mice exhibit a specific disruption in NK cell development resulting in severely reduced numbers of mature NK cells in the periphery[310]. *Nfil3*$^{-/-}$ mice were analyzed by flow cytometry at birth. Nfil3-deficient pups had a significantly reduced population of ELCs when compared to the WT (Figure 25c).

T-bet (T-box transcription factor, encoded by *Tbx21*) is another transcription factor that has been highly involved in NK cell development and maturation. Together with Eomes, T-bet has been shown to instruct the development of distinct NK lineages and control key checkpoints of their maturation[171,206]. However, when analyzing NB from *Tbx21*$^{-/-}$, we saw no distinct phenotype from the WT since both models had similar levels of ELCs in their epidermis (Figure 25d).

Altogether these results shed some light into the transcriptomic requirements of this specific population. ELCs are independent of RORγt and T-bet but dependent of Runx3 and Nfil3. Yet, the exact mechanism behind this dependency remains unknown.

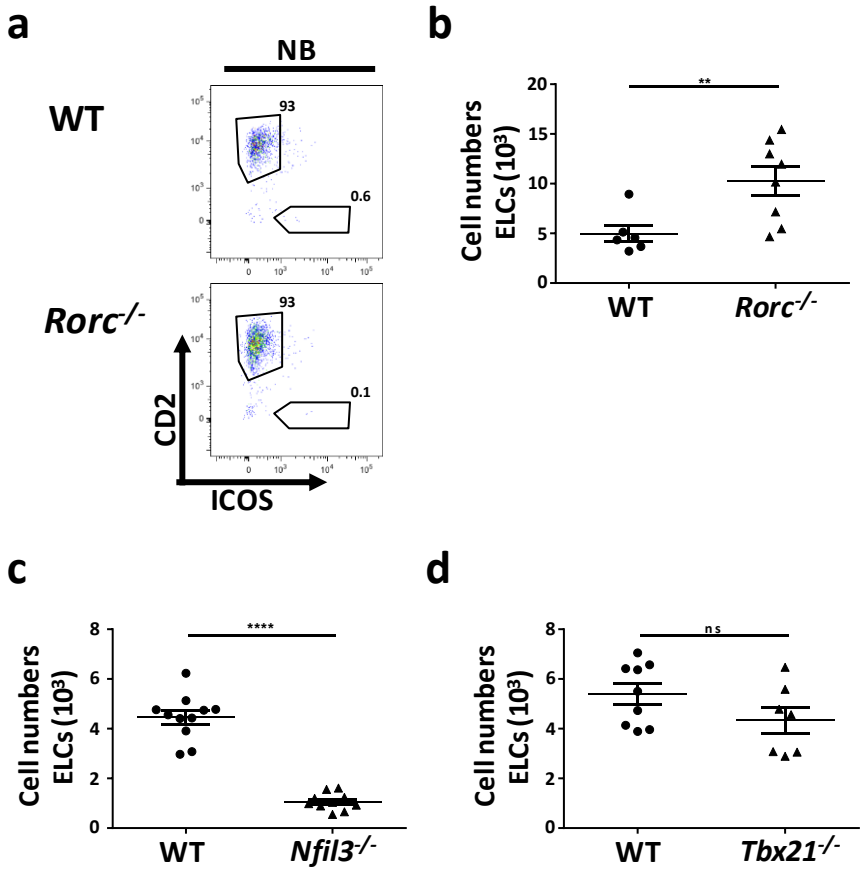


Figure 25. ELCs are independent of RORγt and T-bet but dependent on Nfil3.

a. Flow cytometry of the ELC population in NB from both WT and *Rorc*$^{-/-}$. **b.** Dot plot identifies the absolute numbers between both mouse models. Each data point represents an individual embryo. Data were obtained from n=6 (WT) and n=8 (*Rorc*$^{-/-}$) embryos from 2 independent experiments. **c.** Dot plot identifies the absolute numbers between WT (n=11) and *Nfil3*$^{-/-}$ (n=10) from 3 independent experiments **d.** Dot plot shows the absolute numbers between WT (n=9) and *Tbx21*$^{-/-}$ (n=7) from 2 independent experiments.

DISCUSSION

5. Discussion

Cutaneous lymphoid populations have been extensively studied under both steady state and inflammatory conditions. DETCs are the major lymphoid cell type in the murine epidermis in the steady state[56], while following inflammation epidermal $CD8^+$ T cells become part of the immune pool found in this tissue[55]. In contrast, the dermis is populated by different types of T cells, including αβ and γδ T cells, NK cells and the recently-described ILC2[1,50,66].

Here we add to the knowledge of the epidermal immune compartment by characterizing a new lymphoid population that is present at low frequencies in the adult WT epidermis, but accumulates in the epidermis of adult *Rag*-deficient models or athymic mice. However, this population has shown to be heterogeneous, being $ICOS^+$ or $CD2^+$. While $ICOS^+$ fraction are the ILC2 reported by Roediger *et al*, the $CD2^+$ fraction is our population of interest and named ELCs (Epidermal Lymphoid Cells).

Interestingly, the ELC population shares some molecular cues with DETCs by being IL2γ and IL15Rα dependent. In human skin, the primary cellular source of IL-15 is keratinocytes, constitutively expressing mRNA and producing the protein in response to exogenous stimuli[311–313]. In addition, in mice, IL-15 has been shown to promote the growth of DETCs by a mechanism involving the beta and gamma c-chains of the IL-2 receptor[273]. Thus, interaction with keratinocytes might be in place to support the development and/or survival of ELCs in an IL15 dependent manner. We also hypothesize that DETCs and ELCs might compete for IL-15 signalling, resulting in the low frequencies of ELCs found in the adult epidermis.

Furthermore, the ELC population also has similar homeostasis as DETCs and different from the ILC compartment, since these cells have shown to be radioresistant. Also, upon parabiosis, the ELCs have shown to be independent from circulating progenitors, similarly to DETCs. These homeostatic cues raise the possibility that ELCs, like DETCs[314], might be restricted to an embryonic wave of progenitors that seeds the epidermis for life.

Extrathymic education has been a much debated topic where several studies have been published showing that maturation of T cells outside the thymus can occur[315,316],

especially since athymic mice have T cells[128]. Since this lymphoid population was expanded in the *Rag1*$^{-/-}$ and nude epidermis, plus exhibited characteristics restricted to the T cell-lineage, we hypothesize that the ELC population could be a pool of T cell progenitors in the adult mouse. It has been reported that cells belonging to T cell-lineage would express CD3 intracellularly[281–283], and variable proportions of the ELC population were positive for cytoplasmic CD3. In addition, the expression of pTα is also exquisitely T lineage-specific and occurs in pro-T cells outside the thymus as well as at sites that support extrathymic T cell development[285]. Unexpectedly, we could also observe some expression of pTα in the ELC population. Both these characteristics in this population are in accordance with our T cell progenitor premise. Therefore, we hypothesized that either this lymphoid population is a pool of T cell progenitors on their way along the T cell differentiation pathway or is a heterogeneous pool of cells with a fraction of T cell progenitors together with other unknown cell types.

It is quite puzzling that these cells exhibit a thymic DN stage-like phenotype and do not develop further with age. We questioned whether the presence of DETCs, by cytokine competition or niche occupation, would be limiting the development of the ELC population. One possibility, due to the CD2 expression in this lymphoid population, would be that these cells were CIPs as described by Dejbakhsh-Jones and collaborators[133]. In concordance with this hypothesis is the fact that a small population of Thy1^{high}CD2^{+} cells, similar to CIPs found in WT, have been shown to exist in the bone marrow of nude mice[317]. If true, these intermediate progenitors should be able to develop through an extrathymic pathway into mature T cells; however, using a *Rag*-inducible mouse model that enabled the reinstatement of normal thymocyte development, ELCs retained their existing phenotype. One possible explanation is that the adult skin simply does not provide the correct environment for these cells to further differentiate.

ELCs have shown to be radioresistant and independent of circulating progenitors suggesting that these cells colonize the epidermis earlier than adult life, similarly to DETCs[314]. When analyzing earlier time points, before and after birth, we observed that ELCs are present in both *Rag1*$^{-/-}$ and WT epidermis as early as E17.5 together with DETCs. Throughout development of the mouse, DETC frequency increases until adulthood in the WT epidermis, while the ELC population reaches almost undetectable levels.

Contrastingly, in the *Rag*-deficient mice ELCs keep expanding, only decreasing slightly in the adult mice. The reason for the decrease in frequency is currently unknown. We hypothesize that the disappearance of ELCs in the WT postnatal epidermis might be due to direct competition with DETCs for niche occupation or cytokine requirements. Therefore, keratinocyte-derived IL-15 arises as a possible candidate for the cytokine competition theory drawn between DETCs and ELCs. Nevertheless, localization data of the DETCs and ELCs over the NB epidermis could shed some light in this possible proximity and therefore competition.

In addition, similar to the adult data, embryonic ELCs also shared a variable expression of pTα, however levels of expression of CD3ε in their cytoplasm were greatly reduced. Cytoplasmic CD3ε can be detected in thymocytes as early as E13.0 (two days before expression of the TCRβ chain) and it is has been reported to be detected from DN2 to DN3. While some DN2 might be heterogeneous for its expression, by DN3 it is completely established[282]. In addition, our fate mapping model has been reported to show pTα expression as early as DN1[248]. Thus, we hypothesize that the ELCs in late embryogenesis are less developed than the one in the adult epidermis, being the embryonic population the equivalent of the DN1 stage and the adult population more closely related to a stage after DN3.

Taken together, these findings contributed to our hypothesis that this population does seem to belong to an early stage in the T cell lineage. However, and similarly to what we found with the *Rag*-inducible model, even before birth the embryonic skin does not seem to provide the correct environment for the progenitors to develop. In order to further study the nature of these cells and understand their true potential we decided to purify the newborn population and proceed with *in vitro* and *ex vivo* approaches. Nevertheless, upon co-culture with stromal cells supplemented with cytokines the ELC population failed to further develop, maintaining its phenotype.

For many years, researchers have used *ex vivo* approaches to analyze the potential of possible T cell progenitors. By depleting the hematopoietic compartment and culturing thymic lobes, potential progenitors can be introduced to the stromal environment. Without having to add any extra cytokines it is believed that this system is a less artificial model that reproduces more closely what happens *in vivo* and throughout development.

Therefore we decided that FTOC would be a good system where we could test the potential of the ELC population. Nonetheless, while thymic DN could proceed with the expected T cell development that can be found in the *in vivo* thymus, ELCs maintained the same phenotype throughout the culture period. Similarly to what we found in the stromal culture, no upregulation of mature T cell markers such as CD3 or TCR were observed. Of note that a fraction of $CD4^+$ cells was detected, yet no maturation markers followed the CD4 acquisition, as such it is impossible for us to assume that they are T cells.

Even though both adult and embryonic/newborn ELCs exhibit T lineage characteristics such as surface and intracellular markers, they were unable to develop as mature T cells under FTOC conditions, stromal culture or in our *Rag*-inducible model. Taking all of these results together we need to conclude that ELCs are not T cell progenitors.

The true nature of ELCs remains unknown; however as lymphoid cells, this population could be related to the ILC family. Yet, the phenotype of the ELC population is different than *bona fide* ILC, since they lack expression of surface markers characteristic of the different ILC groups, such as ICOS, NKp46, ST2 and c-kit (*data not shown*). More importantly, ELCs are radioresistant, unlike the ILC2 described by Roediger[50] and other ILCs[157,176]. However, we cannot exclude that ELCs could represent a unique and unreported new population of ILCs with unique homeostatic features that could be conferred by the environment where they reside. Accordingly, all immune populations residing in the epidermis, such as DETCs and LCs, are relatively resistant to depletion by radiation and renew themselves locally[278,279,318].

Nevertheless, it is important to note that a combination of CD2 and Thy1 as lymphoid markers can be shared by other lineages other than T cells, such as NK cells. In a phenotyping approach of the NB population, we found that the ELCs in both WT and *Rag1*$^{-/-}$ exhibit expression of NK markers such as CD49b and NK1.1. Additionally, other members of the CD2 family, previously reported to be expressed by NKs[301,319], such as CD244 and CD48 were also expressed by this population. The only known CD244-binding molecule is CD48. While CD244 has been reported to be expressed by DETCs[302], CD48 has been reported to be expressed by LCs[305]. Yet, here we show that in the newborn epidermis both CD244 and CD48 are expressed by ELCs, DETCs and LCs. Together this

phenotype supports our new hypothesis of the ELCs being an NK-like population with a possible interaction among themselves or with the other immune sentinels present in the epidermis.

Moreover, IL2Rβ had a low expression on populations from both WT and *Rag1*$^{-/-}$ mouse models at the NB stage. Previous studies have shown that depletion of either this receptor, IL-15 or IL-15Rα blocks NK development[274–276]. Therefore, in the future we hope to address the impact of IL-15 on the ELC population before or right after birth, to further understand their possibly dependency on this signalling pathway.

Other than cytokine receptor profiling, cytokine production itself can also shed light on the nature of a cell. For example T cells and the newly discovered family of ILCs have been classified based on their cytokine profile[180]. Transcriptomic analysis by NanoString of the NB population highlighted several cytokines of interest that where then addressed experimentally under steady state and stimulatory conditions. We observed that under non-stimulatory conditions ELCs failed to produce any cytokine, however after stimulation, both WT and *Rag1*$^{-/-}$ populations exhibited production of IFN-γ and IL-2. This cytokine profile fits with the NK-like hypothesis since NKs are known to have a profile similar to T_h1[320]. We also hypothesize that the cytokines produced can act on an autocrine or paracrine mechanism in order to further activate cells and be involved in tolerogenic functions in the epidermis.

In addition, another way to classify different cell types is through their expression profile and/or developmental dependency of transcription factors. By analyzing late embryogenesis or newborn time points of deficient mouse models, we observed that the ELC population is dependent on Runx3 and Nfil3. Both transcription factors have been reported in the literature to be highly involved in development and maturation of NK cells[308–310]; however none of them is specific to this lineage. In the future we hope to complement our studies by addressing the EOMES expression to further strengthen our hypothesis of ELCs being an NK-like population.

In conclusion, we are still not sure about the true nature of these cells and their functions. However we do know that they belong to the lymphoid lineage and they are neither T cells or T cell progenitors, nor the ILC2 found previously in the skin by others. We speculate that due to the phenotype and time frame where these cells appear in

higher frequencies in the WT epidermis they might be involved in interactions with the other immune and non-immune cells in the epidermis. We hypothesize that both DETCs and ELCs compete for the IL-15 produced by keratinocytes, making them competitors for the same niche. In addition, we also dare to contemplate the hypothesis of a potential cooperative role of ELCs alongside LCs and DETCs in supporting the establishment of a mature epidermal immune system at birth. Of note that skin-resident DCs have recently been shown to have an active role in remodeling the skin microbiome[321]. Therefore, we hypothesize that these three immune compartments might work together on a tolerogenic role towards skin microbiota upon birth and during the first days of life. A possible signalling pathway involved in this function could be through CD244/CD48 expressed by ELCs, LCs and DETCS, but additional investigations would be required in order to further understand the mechanisms behind such hypothetical partnership. Plus, even though the latest phenotype characterization, as well as molecular and trancriptomic cues, point towards an NK-like population, more experimental approaches would have to be addressed in order finally understand the nature and function of this newly discovered population.

The roles and lineage relations of the ELC population remain an enigma at this stage, and should prove a productive avenue for future research into immunity in the skin.

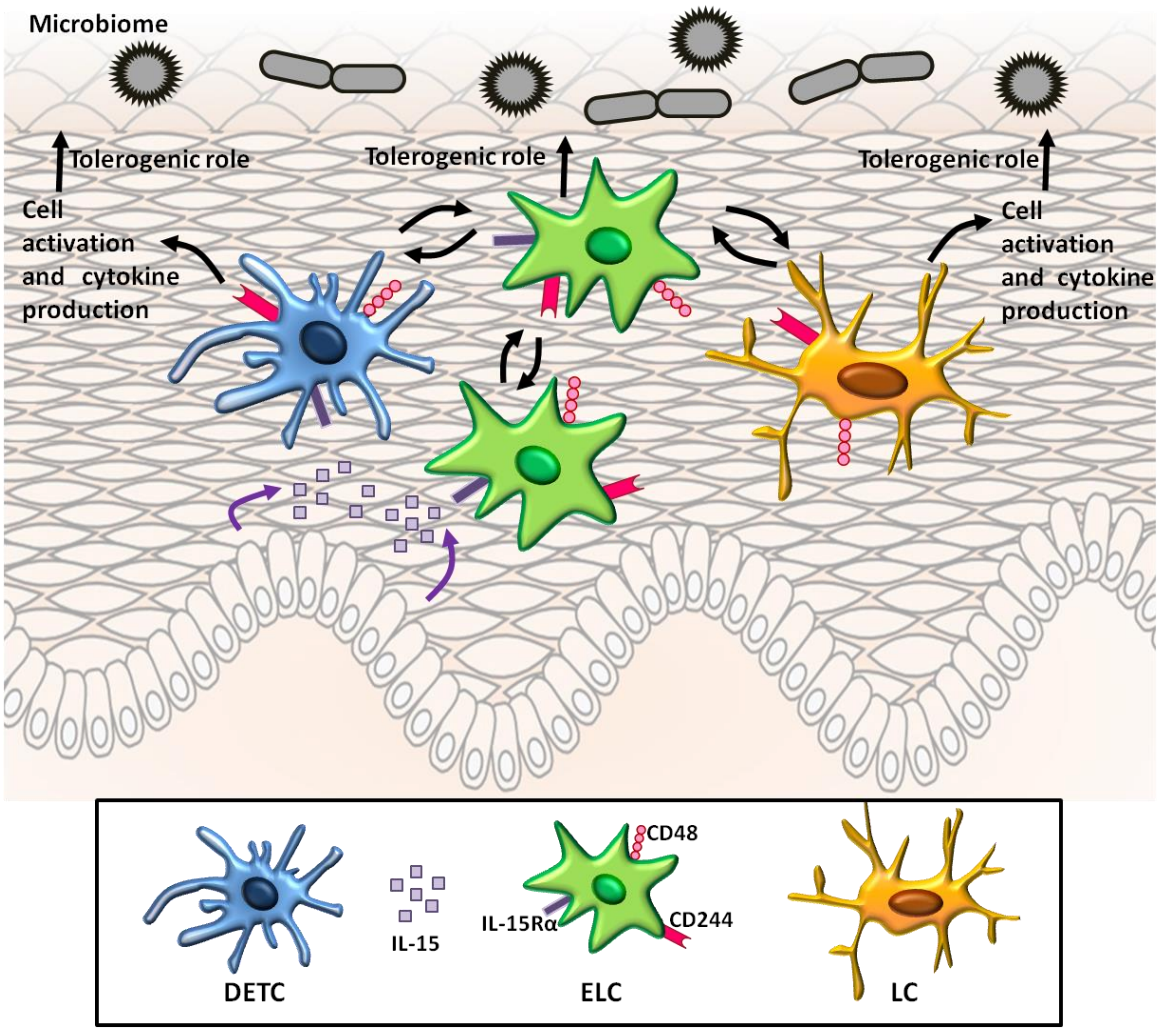


Figure 26. Suggested scheme of interaction of ELCs in the epidermis.

IL-15 produced by keratinocytes interacts with the IL-15Rα, expressed by DETCs and possibly expressed by ELCs, resulting in the proliferation and possible competition between the two populations. Since CD244 and its ligand CD48 are highly expressed by DETCs, ELCs and LCs we propose the above mechanism of interaction. ELCs could interact with all of these cell types leading to their activation and cytokine production. This activation state could have a role in tolerance upon colonization of the newborn epidermis by its microbiome.

SUPPLEMENTARY FIGURES

6. Supplementary figures

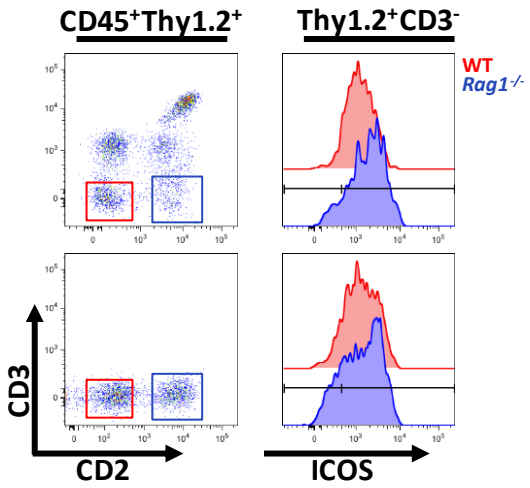


Figure S1. Thy1^{+} population in the dermis.

Flow cytometry of 8wo mouse dermal cell suspension. Gating strategy and histograms to identify ICOS expression across the two populations Thy1^{+}, CD3^{-}CD2^{-} and CD3^{-}CD2^{+} in both WT and *Rag1*$^{-/-}$ models are shown. Representative data from n>5.

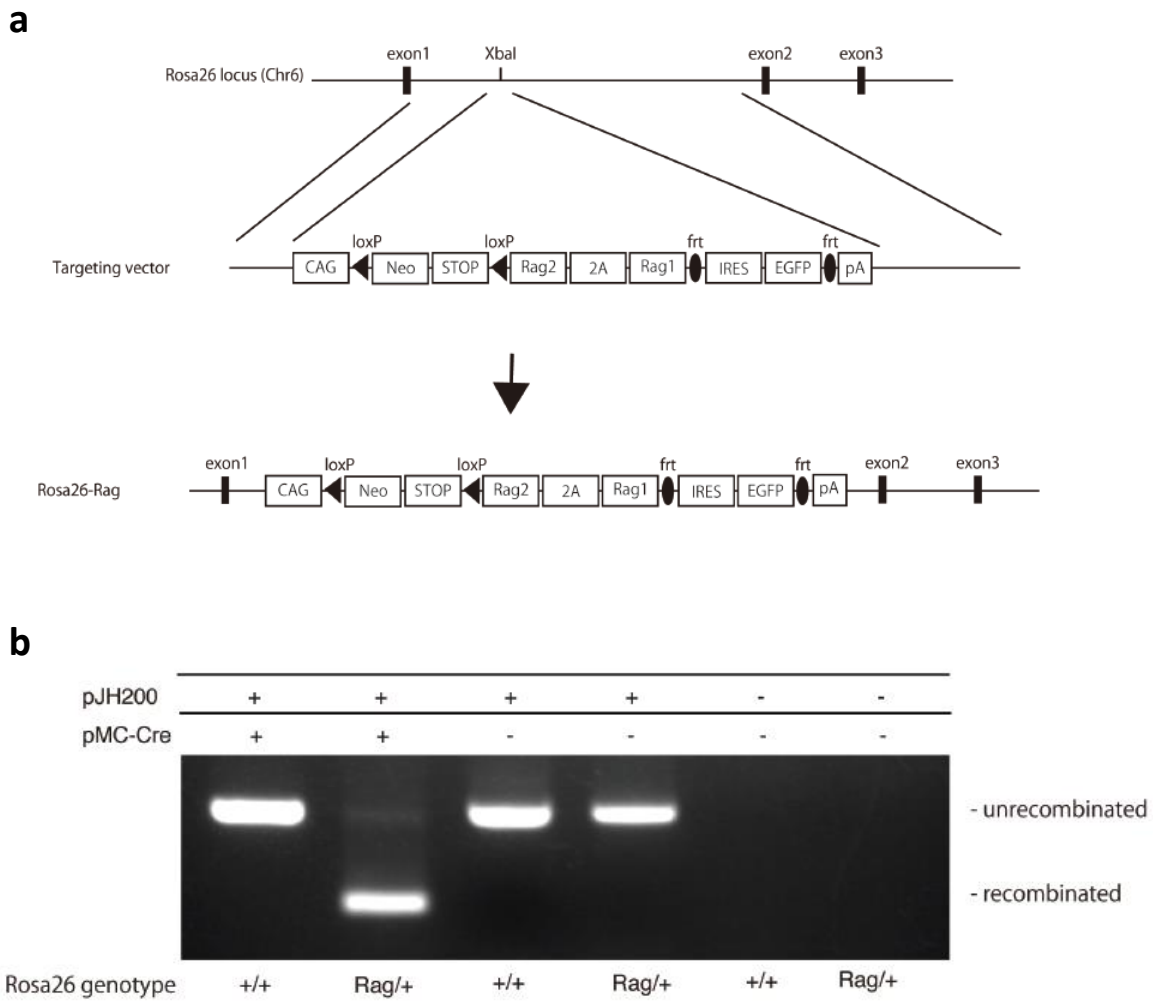


Figure S2. Generation of *Rosa26-Rag* mouse strain.

a. Strategy for generation of *Rosa26-Rag* mouse strain by a "knock-in" insertion of *Rag2-2A-Rag1* cDNA fragment into the *Rosa26* locus. Structures of the WT *Rosa26* locus, the targeting vector, and *Rosa26-Rag* locus were shown. **b.** Analyses of *Rag* activity by *in* vitro recombination assay. PCR assay was performed using feeder cells of the indicated genotype, which were transfected with pJH200 recombination template vector with or without Cre expression vector, pMC-Cre.

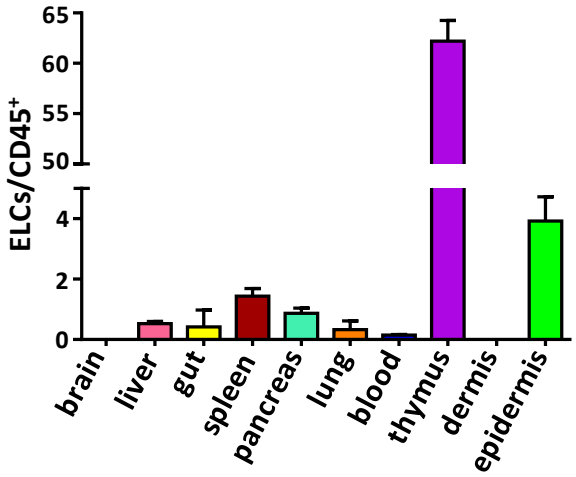


Figure S3. Screening of WT NB for Thy1^{+}CD3^{-}CD2^{+} populations.

Flow cytometry of brain, liver, gut, spleen, pancreas, lung, blood, thymus, dermis and epidermis of WT NB was performed. Bar graphs represent the frequency of Thy1^{+}CD3^{-}CD2^{+} cells among the hematopoietic compartment of the different tissues in the WT NB (n>3).

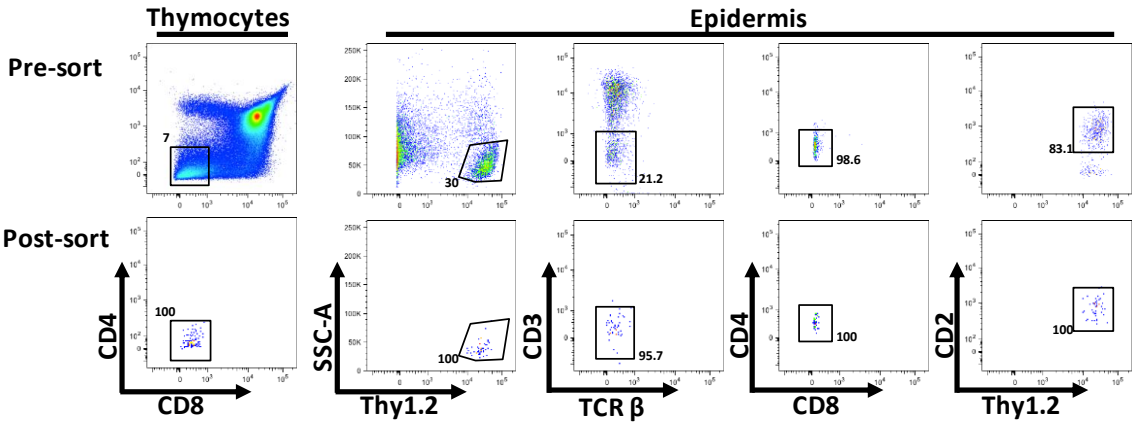


Figure S4. Sort strategy for FTOC system.

Gating scheme and post-sort analysis of thymic DN ($CD4^-CD8^-$) and ELCs from WT NB mice is shown.

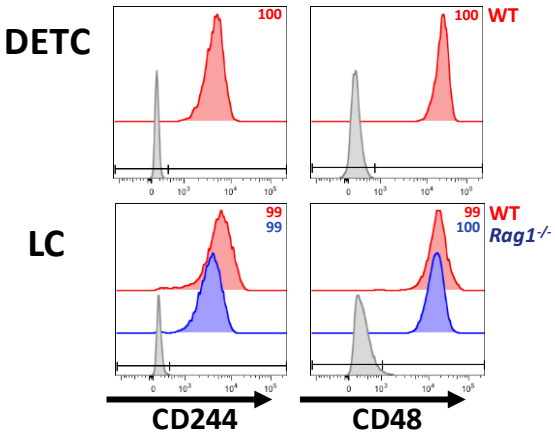


Figure S5. Neonatal DETCs and LCs express CD244 and CD48.

Representative plots of flow cytometry analysis of epidermal cell suspensions from of WT and *Rag1*$^{-/-}$ mice. DETC were gated as CD45^{+}Thy1^{+}CD3^{+} cells while LC were gated as CD45^{+}Thy1^{-}CD11b^{+} and analyzed for their level of expression of CD244 and CD48. Representative data of n=3.

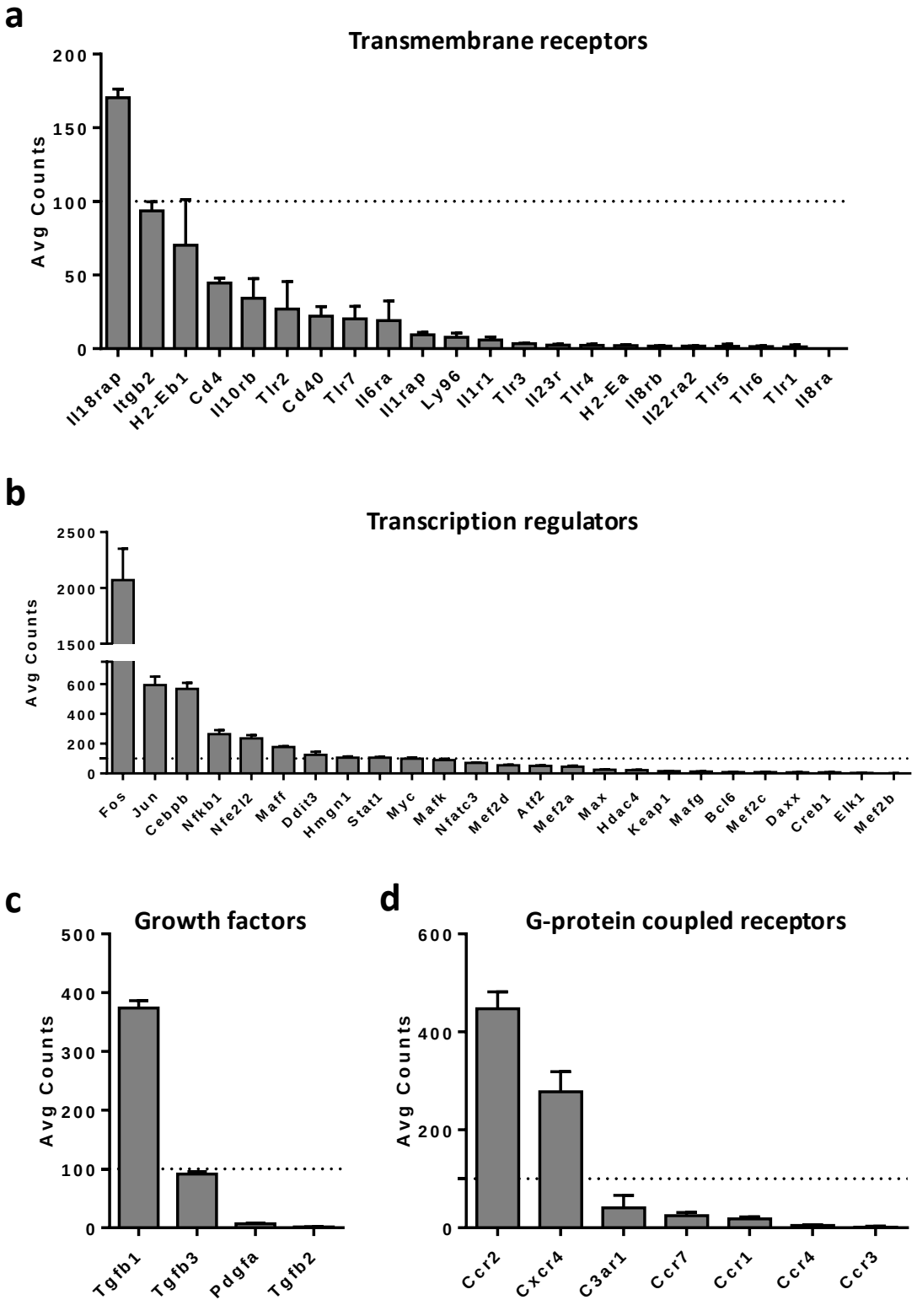


Figure S6. NanoString data set A.

NanoString data on transmembrane receptors (a), transcription regulators (b), growth factors (c) and G-protein coupled receptors (d) of purified ELCs from NB WT. n=4.

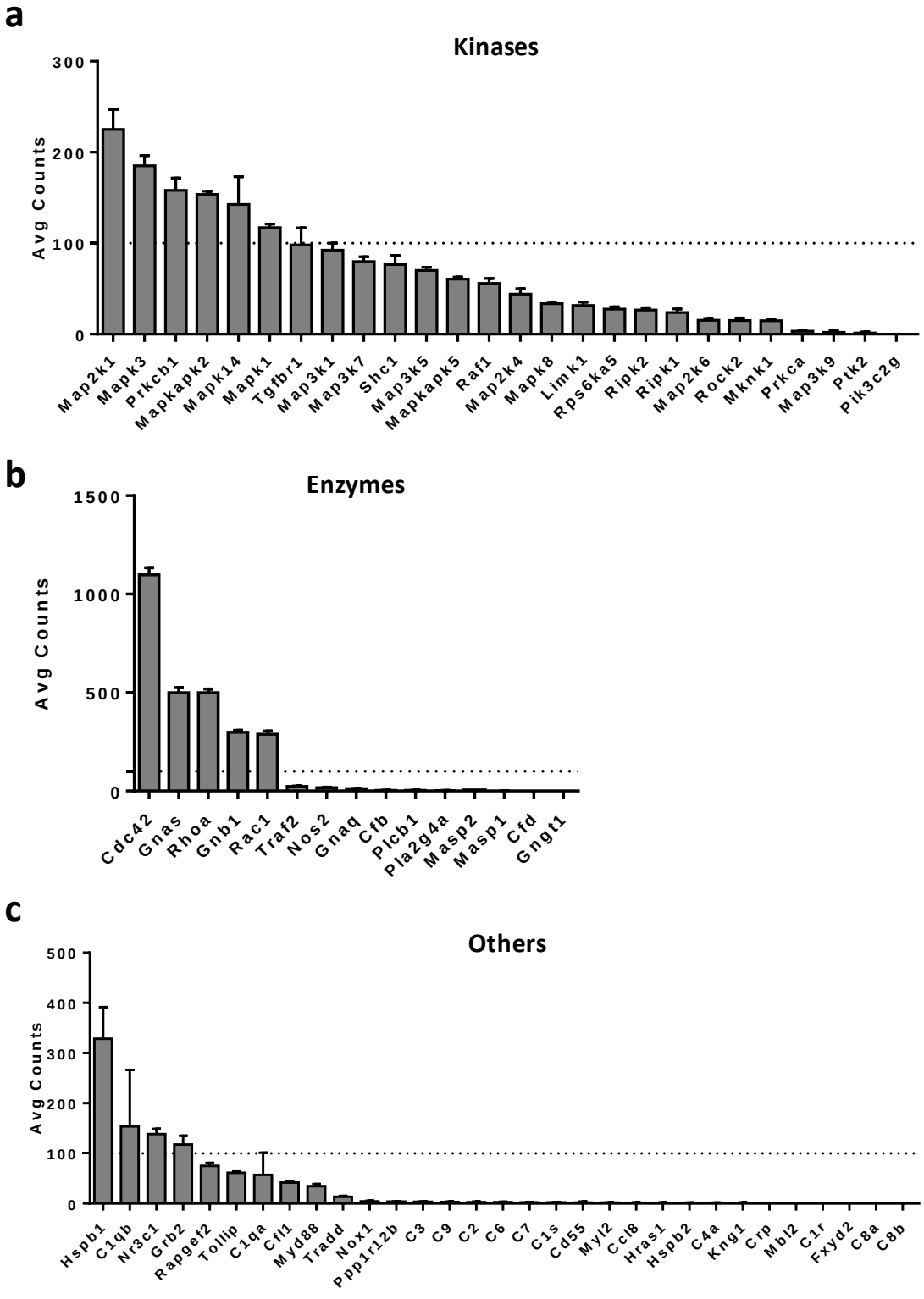


Figure S7. NanoString data set B.

NanoString data on kinases (a), enzymes (b) and others (c) of purified ELCs from NB WT. n=4.

Table 1. Genes analyzed by NanoString.

Class	Cytokine	Enzyme	G-protein coupled receptor	Growth factor	Kinase	Other	Transcription regulator	Transmembrane receptor
Genes	*Ccl11*	*Cdc42*	*C3ar1*	*Pdgfa*	*Limk1*	*C1qa*	*Creb1*	*Cd4*
	Ccl17	*Cfb*	*Ccr1*	*Tgfb1*	*Map2k1*	*C1qb*	*Atf2*	*Cd40*
	Ccl19	*Cfd*	*Ccr2*	*Tgfb2*	*Map2k4*	*C1r*	*Bcl6*	*H2-Ea*
	Ccl2	*Gnaq*	*Ccr3*	*Tgfb3*	*Map2k6*	*C1s*	*Cebpb*	*H2-Eb1*
	Ccl21b	*Gnas*	*Ccr4*		*Map3k1*	*C2*	*Daxx*	*Il10rb*
	Ccl22	*Gnb1*	*Ccr7*		*Map3k5*	*C3*	*Ddit3*	*Il18rap*
	Ccl24	*Gngt1*	*Cxcr4*		*Map3k7*	*C4a*	*Elk1*	*Il1r1*
	Ccl3	*Masp1*			*Map3k9*	*C6*	*Fos*	*Il1rap*
	Ccl4	*Masp2*			*Mapk1*	*C7*	*Hdac4*	*Il22ra2*
	Ccl5	*Nos2*			*Mapk14*	*C8a*	*Hmgn1*	*Il23r*
	Ccl7	*Pla2g4a*			*Mapk3*	*C8b*	*Jun*	*Il6ra*
	Cd40lg	*Plcb1*			*Mapk8*	*C9*	*Keap1*	*Il8ra*
	Csf1	*Rac1*			*Mapkapk*	*Ccl8*	*Maff*	*Il8rb*
	Csf2	*Rhoa*			*Mapkapk*	*Cd55*	*Mafg*	*Itgb2*
	Csf3	*Traf2*			*Mknk1*	*Cfl1*	*Mafk*	*Ly96*
	Cxcl1				*Pik3c2g*	*Crp*	*Max*	*Tlr1*
	Cxcl10				*Prkca*	*Fxyd2*	*Mef2a*	*Tlr2*
	Cxcl2				*Prkcb1*	*Grb2*	*Mef2b*	*Tlr3*
	Cxcl3				*Ptk2*	*Hras1*	*Mef2c*	*Tlr4*
	Cxcl5				*Raf1*	*Hspb1*	*Mef2d*	*Tlr5*
	Cxcl9				*Ripk1*	*Hspb2*	*Myc*	*Tlr6*
	Fasl				*Ripk2*	*Kng1*	*Nfatc3*	*Tlr7*
	Hc				*Rock2*	*Mbl2*	*Nfe2l2*	
	Ifna1				*Rps6ka5*	*Myd88*	*Nfkb1*	
	Ifnb1				*Shc1*	*Myl2*	*Stat1*	
	Ifng				*Tgfbr1*	*Nox1*		
	Il10					*Nr3c1*		
	Il11					*Ppp1r12b*		
	Il12a					*Rapgef2*		
	Il12b					*Tollip*		
	Il13					*Tradd*		
	Il15							
	Il18							
	Il1a							
	Il1b							
	Il1rn							
	Il2							
	Il22							
	Il23a							
	Il3							
	Il4							
	Il5							
	Il6							
	Il7							
	Il9							
	Lta							
	Ltb							
	Tnf							
	Tnfsf14							

REFERENCES

7. References

1. Nestle, F. O., Di Meglio, P., Qin, J.-Z. & Nickoloff, B. J. Skin immune sentinels in health and disease. *Nat. Rev. Immunol.* **9,** 679–91 (2009).
2. Grice, E. a & Segre, J. a. The skin microbiome. *Nat. Rev. Microbiol.* **9,** 244–53 (2011).
3. Belkaid, Y. & Segre, J. A. Dialogue between skin microbiota and immunity. *Sci. Rev.* **346,** 954–960 (2014).
4. Proksch, E., Brandner, J. M. & Jensen, J.-M. The skin: an indispensable barrier. *Exp. Dermatol.* **17,** 1063–1072 (2008).
5. Madison, K. C. Barrier Function of the Skin: 'La Raison d'Être' of the Epidermis. *J. Invest. Dermatol.* **121,** 231–241 (2003).
6. Mackay, L. K. *et al.* The developmental pathway for CD103(+)CD8+ tissue-resident memory T cells of skin. *Nat. Immunol.* **14,** 1294–301 (2013).
7. Mueller, S. N., Zaid, A. & Carbone, F. R. Tissue-resident T cells: dynamic players in skin immunity. *Front. Immunol.* **5,** 332 (2014).
8. Salmon, J. K., Armstrong, C. A. & Ansel, J. C. Conferences and Reviews The Skin as an Immune Organ. *West J. Med.* **160,** 142–152 (1994).
9. Richmond, J. M. & Harris, J. E. Immunology and Skin in Health and Disease. *Cold Spring Harb. Perspect. Med.* **4,** (2014).
10. M'Boneko, V. & Merker, H.-J. Development and Morphology of the Periderm of Mouse Embryos (Days 9–12 of Gestation). *Cells Tissues Organs* **133,** 325–336 (1988).
11. Holbrook, K. A. & Odland, G. F. The fine structure of developing human epidermis light, scanning, and transmission electron microscopy of the periderm. *J. Invest. Dermatol.* **65,** 16–38 (1975).
12. Serri, F. Montagna, W. Mescon, H. Studies of the skin of the fetus and the child. *J. Invest. Dermatol.* **39,** 199–217 (1962).
13. Visscher, M. & Narendran, V. The Ontogeny of Skin. *Adv. wound care* **3,** 291–303 (2014).
14. Kubo, A., Nagao, K., Yokouchi, M., Sasaki, H. & Amagai, M. External antigen uptake by Langerhans cells with reorganization of epidermal tight junction barriers. *J. Exp. Med.* **206,** 2937–2946 (2009).
15. Allan, R. S. *et al.* Epidermal Viral Immunity Induced by CD8alpha+ Dendritic Cells But Not by Langerhans Cells. *Science* **301,** 1925–1928 (2003).
16. Haniffa, M. *et al.* Human Tissues Contain CD141 hi Cross-Presenting Dendritic Cells with Functional Homology to Mouse CD103 + Nonlymphoid Dendritic Cells. *Immunity* **37,** 60–73 (2012).

17. Schlitzer, A. *et al.* IRF4 transcription factor-dependent CD11b+ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. *Immunity* **38,** 970–83 (2013).

18. Persson, E. *et al.* IRF4 Transcription-Factor-Dependent CD103+CD11b+ Dendritic Cells Drive Mucosal T Helper 17 Cell Differentiation. *Immunity* **38,** 958–969 (2013).

19. Ginhoux, F. *et al.* The origin and development of nonlymphoid tissue CD103+ DCs. *J. Exp. Med.* **206,** 3115–3130 (2009).

20. Henri, S. *et al.* CD207+ CD103+ dermal dendritic cells cross-present keratinocyte-derived antigens irrespective of the presence of Langerhans cells. *J. Exp. Med.* **207,** 189–206 (2010).

21. Miller, J. C. *et al.* Deciphering the transcriptional network of the dendritic cell lineage. *Nat. Immunol.* **13,** 888–899 (2012).

22. Farache, J., Zigmond, E., Shakhar, G. & Jung, S. Contributions of dendritic cells and macrophages to intestinal homeostasis and immune defense. *Immunol. Cell Biol.* **91,** 232–9 (2013).

23. McGovern, N. *et al.* Human Dermal CD14+ Cells Are a Transient Population of Monocyte-Derived Macrophages. *Immunity* **41,** 465–477 (2014).

24. Collin, M., Mcgovern, N. & Haniffa, M. Human dendritic cell subsets. *Immunology* **140,** 22–30 (2013).

25. Hunger, R. E. *et al.* Langerhans cells utilize CD1a and langerin to efficiently present nonpeptide antigens to T cells. *J. Clin. Invest.* **113,** 701–708 (2004).

26. Klechevsky, E. *et al.* Functional Specializations of Human Epidermal Langerhans Cells and CD14+ Dermal Dendritic Cells. *Immunity* **29,** 497–510 (2008).

27. Stoitzner, P. *et al.* Langerhans cells cross-present antigen derived from skin. *Proc. Natl. Acad. Sci. U. S. A.* **103,** 7783–7788 (2006).

28. Haley, K. *et al.* Langerhans cells require MyD88-dependent signals for Candida albicans response but not for contact hypersensitivity or migration. *J. Immunol.* **188,** 4334–9 (2012).

29. Igyártó, B. Z. *et al.* Skin-Resident Murine Dendritic Cell Subsets Promote Distinct and Opposing Antigen-Specific T Helper Cell Responses. *Immunity* **35,** 260–272 (2011).

30. Shklovskaya, E. *et al.* Langerhans cells are precommitted to immune tolerance induction. *Proc. Natl. Acad. Sci.* **108,** 18049–18054 (2011).

31. Kaplan, D. H., Jenison, M. C., Saeland, S., Shlomchik, W. D. & Shlomchik, M. J. Epidermal Langerhans cell-deficient mice develop enhanced contact hypersensitivity. *Immunity* **23,** 611–620 (2005).

32. Geissmann, F. *et al.* Accumulation of immature Langerhans cells in human lymph nodes draining chronically inflamed skin. *J. Exp. Med.* **196,** 417–430 (2002).

33. Ochoa, M. T., Loncaric, A., Krutzik, S. R., Becker, T. C. & Modlin, R. L. 'Dermal dendritic cells' comprise two distinct populations: CD1+ dendritic cells and CD209+ macrophages. *J. Invest. Dermatol.* **128,** 2225–2231 (2008).

34. Zaba, L. C., Fuentes-duculan, J., Steinman, R. M., Krueger, J. G. & Lowes, M. a. Normal human dermis contains distinct populations of CD11c+BDCA-1+ dendritic cells and CD163+FXIIIA+ macrophages. **117,** 2517–2525 (2007).

35. Van Furth, R., Nibbering, P. H., van Dissel, J. T. & Diesselhoff-den Dulk, M. M. The characterization, origin, and kinetics of skin macrophages during inflammation. *J. Invest. Dermatol.* **85,** 398–402 (1985).

36. Stratis, A. *et al.* Pathogenic role for skin macrophages in a mouse model of keratinocyte-induced psoriasis-like skin inflammation. *J. Clin. Invest.* **116,** 2094–2104 (2006).

37. Fuentes-Duculan, J. *et al.* A subpopulation of CD163-positive macrophages is classically activated in psoriasis. *J. Invest. Dermatol.* **130,** 2412–2422 (2010).

38. Schaeffer, E. *et al.* Dermal CD14+ Dendritic Cell and Macrophage Infection by Dengue Virus Is Stimulated by Interleukin-4. *J. Invest. Dermatol.* (2015). doi:10.1038/jid.2014.525

39. Chiang, N. *et al.* Infection regulates pro-resolving mediators that lower antibiotic requirements. *Nature* **484,** 524–528 (2012).

40. Egawa, M. *et al.* Inflammatory Monocytes Recruited to Allergic Skin Acquire an Anti-inflammatory M2 Phenotype via Basophil-Derived Interleukin-4. *Immunity* **38,** 570–580 (2013).

41. Kobayashi, M. *et al.* Abrogation of high-affinity IgE receptor-mediated mast cell activation at the effector phase prevents contact hypersensitivity to oxazolone. *J. Invest. Dermatol.* **130,** 725–731 (2010).

42. Torii, Morikawa, S., Harada, T. & Kitamura, Y. Two distinct types of cellular mechanisms in the development of delayed hypersensitivity in mice: requirement of either mast cells or macrophages for elicitation of the response. *Immunology* **78,** 482–490 (1993).

43. Hershko, A. Y. *et al.* Mast Cell Interleukin-2 Production Contributes to Suppression of Chronic Allergic Dermatitis. *Immunity* **35,** 562–571 (2011).

44. Nakamura, Y. *et al.* Staphylococcus δ-toxin induces allergic skin disease by activating mast cells. *Nature* **503,** 397–401 (2013).

45. Shin, K. *et al.* Mouse Mast Cell Tryptase mMCP-6 Is a Critical Link between Adaptive and Innate Immunity in the Chronic Phase of Trichinella spiralis Infection. *J. Immunol.* **180,** 4885–4891 (2008).

46. Galli, S. J. Rethinking the potential roles of mast cells in skin wound healing and bleomycin-induced skin fibrosis. *J. Invest. Dermatol.* **134,** 1802–4 (2014).

47. Luci, C. *et al.* Influence of the transcription factor RORgammat on the development of NKp46+ cell populations in gut and skin. *Nat. Immunol.* **10,** 75–82 (2009).

48. Batista, M. D. *et al.* Skewed distribution of natural killer cells in psoriasis skin lesions. *Exp. Dermatol.* **22,** 64–66 (2013).

49. Ebert, L. M., Meuter, S. & Moser, B. Homing and function of human skin gammadelta T cells and NK cells: relevance for tumor surveillance. *J. Immunol.* **176,** 4331–4336 (2006).

50. Roediger, B. *et al.* Cutaneous immunosurveillance and regulation of inflammation by group 2 innate lymphoid cells. *Nat. Immunol.* **14,** 564–573 (2013).

51. Pantelyushin, S. *et al.* Brief report: Rorγt+ innate lymphocytes and γδ T cells initiate psoriasiform plaque formation in mice. *J. Clin. Invest.* **122,** 2252–6 (2012).

52. Clark, R. a. *et al.* The Vast Majority of CLA+ T Cells Are Resident in Normal Skin. *J. Immunol.* **176,** 4431–4439 (2006).

53. Bos, J. D. *et al.* The skin immune system (SIS): Distribution and Immunophenotype of Lymphocyte Subpopulations in Normal Human Skin. *J. Invest. Dermatol.* **88,** 569–573 (1987).

54. Foster, C. A. *et al.* Human Epidermal T cells predominantely belong to the lineage of alpha/beta T cell receptor. *J. Exp. Med.* **171,** 997–1013 (1990).

55. Gebhardt, T. *et al.* Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus. *Nat. Immunol.* **10,** 524–30 (2009).

56. Tschachler, E. *et al.* Expression of Thy-1 antigen by murine epidermal cells. *J. Invest. Dermatol.* **81,** 282–285 (1983).

57. Bergstresser, P. R., Tigelaar, R. E. & Streilein, J. W. Thy-1 Antigen-Bearing Dendritic Cells in Murine Epidermis are derived from Bone Marrow Precursors. *J. Invest. Dermatol.* **83,** 83–87 (1984).

58. Romani, B. Y. N. *et al.* The Thy-1-bearing cell of murine epidermis. A distinctive leukocyte perhaps related to Natural Killer Cells. *J. Exp. Med.* **161,** 1368–1383 (1985).

59. Bos, J. D. & Kapsenberg, M. L. The Skin immune system: progress in cutaneous biology. *Immunol. Today* **14,** 0–3 (1993).

60. Edele, F. *et al.* Cutting Edge : Instructive Role of Peripheral Tissue Cells in the Imprinting of T Cell Homing Receptor Patterns. *J. Immunol.* **181,** 3745–9 (2008).

61. Mora, J. R. *et al.* Reciprocal and dynamic control of CD8 T cell homing by dendritic cells from skin- and gut-associated lymphoid tissues. *J. Exp. Med.* **201,** 303–16 (2005).

62. Naik, S. *et al.* Compartmentalized Control of Skin. *Science* **337,** 1115–1120 (2012).

63. Jameson, J. *et al.* A role for skin gamma delta T cells in wound repair. *Science* **296,** 747–749 (2002).

64. Girardi, M. *et al.* Resident Skin-specific gamma delta T Cells Provide Local , Nonredundant Regulation of Cutaneous Inflammation. *J. Exp. Med.* **195,** (2002).

65. Kaminski, M. J., Cruz, P. D., Bergstresser, P. R. & Takashima, A. Killing of Skin-derived Tumor Cells by Mouse Dendritic Epidermal T-Cells. *Cancer Res.* **4,** 4014 – 4019 (1993).

66. Sumaria, N. *et al.* Cutaneous immunosurveillance by self-renewing dermal gammadelta T cells. *J. Exp. Med.* **208,** 505–18 (2011).

67. Cai, Y. *et al.* Pivotal role of dermal IL-17-producing γδ T cells in skin inflammation. *Immunity* **35,** 596–610 (2011).

68. Bos, J. *et al.* T-Cell Receptor γδ Bearing Cells in Normal Human Skin. *J. Invest. Dermatol.* **94,** 37–42 (1990).

69. Dupuy, P. *et al.* T-Cell Receptor-γδ Bearing Lymphocytes in Normal and Inflammatory Human Skin. *J. Invest. Dermatol.* **94,** 764–768 (1990).

70. Fujita, M., Miyachi, Y., Nakata, K. & Imamura, S. γδ T-cell receptor-positive cells in human skin. I. Incidence and V-region gene expression in granulomatous skin lesions. *J. Am. Acad. Dermatol.* **28,** 46–50 (1993).

71. Holtmeier, W. *et al.* The TCR-delta repertoire in normal human skin is restricted and distinct from the TCR-delta repertoire in the peripheral blood. *J. Invest. Dermatol.* **116,** 275–80 (2001).

72. Rodriguez, R. S., Pauli, M., Neuhaus, I. & Al, E. Memory regulatory T cells reside in human skin. *J. Clin. Invest.* **124,** 1027–36 (2014).

73. Hogan, B. R. J. *et al.* Protection from Respiratory Virus Infections Can Be Mediated by Antigen-specific CD4+ T Cells That Persist in the Lungs. *J. Exp. Med.* **193,** 981–986 (2001).

74. Stittelaar, K. J. *et al.* Modified vaccinia virus Ankara protects macaques against respiratory challenge with monkeypox virus. *J. Virol.* **79,** 7845–51 (2005).

75. Xu, R., Johnson, a. J., Liggitt, D. & Bevan, M. J. Cellular and Humoral Immunity against Vaccinia Virus Infection of Mice. *J. Immunol.* **172,** 6265–6271 (2004).

76. Gebhardt, T. *et al.* Different patterns of peripheral migration by memory CD4+ and CD8+ T cells. *Nature* **477,** 216–9 (2011).

77. Jiang, X. *et al.* Skin infection generates non-migratory memory CD8+ T(RM) cells providing global skin immunity. *Nature* **483,** 227–31 (2012).

78. Tavian, M. & Péault, B. Embryonic development of the human hematopoietic system. *Int. J. Dev. Biol.* **49,** 243–250 (2005).

79. Orkin, S. H. & Zon, L. I. Hematopoiesis: An Evolving Paradigm for Stem Cell Biology. *Cell* **132,** 631–644 (2008).

80. Müller, A. M., Medvinsky, A., Strouboulis, J., Grosveld, F. & Dzierzakt, E. Development of hematopoietic stem cell activity in the mouse embryo. *Immunity* **1,** 291–301 (1994).

81. Naito, M., Takahashi, K. & Nishikawa, S. Development, differentiation, and maturation of macrophages in the fetal mouse liver. *J. Leukoc. Biol.* **48,** 27–37 (1990).

82. Delassus, S. & Cumano, A. Circulation of hematopoietic progenitors in the mouse embryo. *Immunity* **4,** 97–106 (1996).

83. Adkins, B. *et al.* Early events in T-cell maturation. *Annu. Rev. Immunol.* **5,** 325–65 (1987).

84. Shortman, K. & Wu, L. Early T lymphocyte progenitors. *Annu. Rev. Immunol.* **14,** 29–47 (1996).

85. Ciofani, M. & Zúñiga-Pflücker, J. C. Determining γδ versus αß T cell development. *Nat. Rev. Immunol.* **10,** 657–63 (2010).

86. Lind, E. F., Prockop, S. E., Porritt, H. E. & Petrie, H. T. Mapping precursor movement through the postnatal thymus reveals specific microenvironments supporting defined stages of early lymphoid development. *J. Exp. Med.* **194,** 127–134 (2001).

87. Prockop, S. E. *et al.* Stromal cells provide the matrix for migration of early lymphoid progenitors through the thymic cortex. *J. Immunol.* **169,** 4354–4361 (2002).

88. Porritt, H. E., Gordon, K. & Petrie, H. T. Kinetics of steady-state differentiation and mapping of intrathymic-signaling environments by stem cell transplantation in nonirradiated mice. *J. Exp. Med.* **198,** 957–962 (2003).

89. Norment, a M. & Bevan, M. J. Role of chemokines in thymocyte development. *Semin. Immunol.* **12,** 445–455 (2000).

90. Porritt, H. E. *et al.* Heterogeneity among DN1 prothymocytes reveals multiple progenitors with different capacities to generate T cell and non-T cell lineages. *Immunity* **20,** 735–745 (2004).

91. Allman, D. *et al.* Thymopoiesis independent of common lymphoid progenitors. *Nat. Immunol.* **4,** 168–174 (2003).

92. Benz, C., Martins, V. C., Radtke, F. & Bleul, C. C. The stream of precursors that colonizes the thymus proceeds selectively through the early T lineage precursor stage of T cell development. *J. Exp. Med.* **205,** 1187–1199 (2008).

93. Luc, S. *et al.* The Earliest Thymic T Cell Progenitors Sustain B Cell and Myeloid Lineage Potentials. *Nat. Immunol.* **13,** 412–9 (2011).

94. Carlyle, J. R. *et al.* Identification of a novel developmental stage marking lineage commitment of progenitor thymocytes. *J. Exp. Med.* **186,** 173–182 (1997).

95. Luche, H. *et al.* The earliest intrathymic precursors of CD8α+ thymic dendritic cells correspond to myeloid-type double-negative 1c cells. *Eur. J. Immunol.* **41,** 2165–2175 (2011).

96. Falk, I., Nerz, G., Haidl, I., Krotkova, A. & Eichmann, K. Immature thymocytes that fail to express TCRbeta and/or TCRgammadelta proteins die by apoptotic cell death in the CD44-CD25- (DN4) subset. *Eur. J. Immunol.* **31,** 3308–3317 (2001).

97. Michie, A. M. & Zúñiga-Pflücker, J. C. Regulation of thymocyte differentiation: pre-TCR signals and β-selection. *Semin. Immunol.* **14,** 351–358 (2002).

98. Xiong, J., Armato, M. a. & Yankee, T. M. Immature single-positive CD8+ thymocytes represent the transition from Notch-dependent to Notchindependent T-cell development. *Int. Immunol.* **23,** 55–64 (2011).

99. Lu, M. *et al.* The earliest thymic progenitors in adults are restricted to T, NK, and dendritic cell lineage and have a potential to form more diverse TCRbeta chains than fetal progenitors. *J. Immunol.* **175,** 5848–5856 (2005).

100. Ge, Q. & Chen, W. F. Phenotypic identification of the subgroups of murine T-cell receptor alphabeta+ CD4+ CD8- thymocytes and its implication in the late stage of thymocyte development. *Immunology* **97,** 665–671 (1999).

101. Gabor, M. J., Godfrey, D. I. & Scollay, R. Recent thymic emigrants are distinct from most medullary thymocytes. *Eur. J. Immunol.* **27,** 2010–2015 (1997).

102. Huang, E. Y., Gallegos, A. M., Richards, S. M., Lehar, S. M. & Bevan, M. J. Surface expression of Notch1 on thymocytes: correlation with the double-negative to double-positive transition. *J. Immunol.* **171,** 2296–2304 (2003).

103. Harman, B. C., Jenkinson, E. J. & Anderson, G. Microenvironmental regulation of Notch signalling in T cell development. *Semin. Immunol.* **15,** 91–97 (2003).

104. Felli, M. P. *et al.* Expression pattern of Notch1, 2 and 3 and Jagged1 and 2 in lymphoid and stromal thymus components: Distinct ligand-receptor interactions in intrathymic T cell development. *Int. Immunol.* **11,** 1017–1025 (1999).

105. Terszowski, G. *et al.* Evidence for a functional second thymus in mice. *Science* **312,** 284–287 (2006).

106. Dooley, J., Erickson, M., Gillard, G. O. & Farr, A. G. Cervical thymus in the mouse. *J. Immunol.* **176,** 6484–6490 (2006).

107. Norris, E. The morphogenesis and histogenesis of the thymus gland in man: in which the origin of the Hassall's corpuscles of the human thymus is discovered. *Contrib Embryol* **27,** (1938).

108. Jv, K. M. & Devi, S. Morphological features of human thymus glands from foetal to old age. *Int. J. Biol. Med. Res.* **3,** 1502–1505 (2012).

109. Loney, D. A. & Bauman, N. M. Ectopic cervical thymic masses in infants. *Int. J. Pediatr. Otorhinolaryngol.* **43,** 77–84 (1998).

110. Sang Ling Wu, Gupta, D. & Connelly, J. Adult ectopic thymus adjacent to thyroid and parathyroid. *Arch. Pathol. Lab. Med.* **125,** 842–843 (2001).

111. Cornu, A. S., Moerman, M., Bonte, K. & Vermeersch, H. Ectopic cervical thymus: case report and review of the literature. *Acta Otorhinolaryngol. Belgica* **55,** 295–8 (2001).

112. Pai, I. *et al.* Ectopic thymus presenting as a subglottic mass: diagnostic and management dilemmas. *Int. J. Pediatr. Otorhinolaryngol.* **69,** 573–6 (2005).

113. Zúñiga-Pflücker, J. C. T-cell development made simple. *Nat. Rev. Immunol.* **4,** 67–72 (2004).

114. Rocha, B., Guy-Grand, D. & Vassalli, P. Extrathymic T cell differentiation. *Curr. Opin. Immunol.* **7,** 235–242 (1995).

115. Park, S. H. *et al.* Selection and expansion of CD8alpha/alpha+ T cell receptor alpha/beta+ intestinal intraepithelial lymphocytes in the absence of both classical

major histocompatibility complex class I and nonclassical CD1 molecules. *J. Exp. Med.* **190,** 885–890 (1999).

116. Gapin, L., Cheroutre, H. & Kronenberg, M. Cutting edge: TCR alpha beta+ CD8 alpha alpha+ T cells are found in intestinal intraepithelial lymphocytes of mice that lack classical MHC class I molecules. *J. Immunol.* **163,** 4100–4104 (1999).

117. Das, B. G. & Janeway, C. A. Development of CD8alpha/alpha and CD8alpha/beta T cells in Major Histocompatibility Complex Class I – deficient Mice. *J. Exp. Med.* **190,** 881–884 (1999).

118. Rocha, B., Vassalli, P. & Guy-Grand, D. The Vbeta Repertoire of Mouse Gut Homodimeric alpha CD8 + Intraepithelial T Cell Receptor alpha/beta+ Lymphocytes Reveals a Major Extrathymic Pathway of T Cell Differentiation. *J. Exp. Med.* **173,** 2–5 (1991).

119. Malissen, M. *et al.* T cell development in mice lacking the CD3-Zeta/eta gene. *EMBO J.* **12,** 4347–4355 (1993).

120. Hamerman, a, Page, S. T. & Pullen, A. M. Distinct Methylation States of the C8beta Gene in Peripheral T cells and Intraepithelial Lymphocytes. *J. Immunol.* **159,** 1240–6 (1997).

121. Sugahara, S. *et al.* Extrathymic derivation of gut lymphocytes in parabiotic mice. *Immunology* **96,** 57–65 (1999).

122. Lin, T., Matsuzaki, G., Kenai, H. & Nomoto, K. Progenies of fetal thymocytes are the major source of CD4-CD8+ alpha alpha intestinal intraepithelial lymphocytes early in ontogeny. *Eur. J. Immunol.* **24,** 1785–1791 (1994).

123. Lefrançois, L. & Olson, S. A Novel Pathway of Thymus-Directed T Lymphocyte Maturation. *Immunology* **153,** 987–995 (1994).

124. Kanamori, Y. *et al.* Identification of novel lymphoid tissues in murine intestinal mucosa where clusters of c-kit+ IL-7R+ Thy1+ lympho-hemopoietic progenitors develop. *J. Exp. Med.* **184,** 1449–1459 (1996).

125. Saito, H. *et al.* Generation of intestinal T cells from progenitors residing in gut cryptopatches. *Science* **280,** 275–278 (1998).

126. Suzuki, K. *et al.* Gut cryptopatches: Direct evidence of extrathymic anatomical sites for intestinal lymphopoiesis. *Immunity* **13,** 854–855 (2000).

127. Lambolez, F. *et al.* Characterization of T cell differentiation in the murine gut. *J. Exp. Med.* **195,** 437–449 (2002).

128. Guy-Grand, D. *et al.* Extrathymic T Cell Lymphopoiesis: Ontogeny and Contribution to Gut Intraepithelial Lymphocytes in Athymic and Euthymic Mice. *J. Exp. Med.* **197,** 333–341 (2003).

129. Kondo, M., Weissman, I. L. & Akashi, K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. *Cell* **91,** 661–672 (1997).

130. Rodewald, H. R., Kretzschmar, K., Takeda, S., Hohl, C. & Dessing, M. Identification of pro-thymocytes in murine fetal blood: T lineage commitment can precede thymus colonization. *EMBO J.* **13,** 4229–40 (1994).

131. García-Ojeda, M. E., Dejbakhsh-Jones, S., Weissman, I. L. & Strober, S. An alternate pathway for T cell development supported by the bone marrow microenvironment: recapitulation of thymic maturation. *J. Exp. Med.* **187,** 1813–23 (1998).

132. Dejbakhsh-Jones, S. & Strober, S. Identification of an early T cell progenitor for a pathway of T cell maturation in the bone marrow. *Proc. Natl. Acad. Sci. U. S. A.* **96,** 14493–8 (1999).

133. Dejbakhsh-Jones, S., Garcia-Ojeda, M. E., Chatterjea-Matthes, D., Zeng, D. & Strober, S. Clonable progenitors committed to the T lymphocyte lineage in the mouse bone marrow; use of an extrathymic pathway. *Proc. Natl. Acad. Sci. U. S. A.* **98,** 7455–60 (2001).

134. García-Ojeda, M. E. *et al.* Stepwise development of committed progenitors in the bone marrow that generate functional T cells in the absence of the thymus. *J. Immunol.* **175,** 4363–73 (2005).

135. Krueger, A. & von Boehmer, H. Identification of a T lineage-committed progenitor in adult blood. *Immunity* **26,** 105–16 (2007).

136. Kawamoto, H., Ohmura, K. & Katsura, Y. Direct evidence for the commitment of hematopoietic stem cells to T, B and myeloid lineages in murine fetal liver. *Int. Immunol.* **9,** 1011–9 (1997).

137. Kawamoto, H., Ohmura, K., Hattori, N. & Katsura, Y. Hemopoietic progenitors in the murine fetal liver capable of rapidly generating T cells. *J. Immunol.* **158,** 3118–24 (1997).

138. Lancrin, C. *et al.* Major T cell progenitor activity in bone marrow-derived spleen colonies. *J. Exp. Med.* **195,** 919–929 (2002).

139. Arcangeli, M.-L. *et al.* Extrathymic hemopoietic progenitors committed to T cell differentiation in the adult mouse. *J. Immunol.* **174,** 1980–1988 (2005).

140. Maillard, I. *et al.* Notch-dependent T-lineage commitment occurs at extrathymic sites following bone marrow transplantation. *Blood* **107,** 3511–3519 (2006).

141. Gautreau, L. *et al.* Identification of an IL-7-dependent pre-T committed population in the spleen. *J. Immunol.* **179,** 2925–2935 (2007).

142. Cortez, V. S., Robinette, M. L. & Colonna, M. Innate lymphoid cells : new insights into function and development. *Curr. Opin. Immunol.* **32,** 71–77 (2015).

143. Mckenzie, A. N. J., Spits, H. & Eberl, G. Innate Lymphoid Cells in Inflammation and Immunity. *Immunity* **41,** 366–374 (2014).

144. Spits, H. & Cupedo, T. Innate lymphoid cells: emerging insights in development, lineage relationships, and function. *Annu. Rev. Immunol.* **30,** 647–75 (2012).

145. Di Santo, J. P. Natural killer cell developmental pathways: a question of balance. *Annu. Rev. Immunol.* **24,** 257–86 (2006).

146. Cupedo, T. *et al.* Presumptive lymph node organizers are differentially represented in developing mesenteric and peripheral nodes. *J. Immunol.* **173,** 2968–2975 (2004).

147. Mebius, R. E., Rennert, P. & Weissman, I. L. Developing lymph nodes collect CD4+CD3- LTbeta+ cells that can differentiate to APC, NK cells, and follicular cells but not T or B cells. *Immunity* **7,** 493–504 (1997).

148. Van de Pavert, S. a *et al.* Maternal retinoids control type 3 innate lymphoid cells and set the offspring immunity. *Nature* **508,** 123–7 (2014).

149. Kiessling, R., Klein, E., Pross, H. & Wigzell, H. 'Natural' killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur. J. Immunol.* **5,** 117–121 (1975).

150. Boos, M. D., Yokota, Y., Eberl, G. & Kee, B. L. Mature natural killer cell and lymphoid tissue-inducing cell development requires Id2-mediated suppression of E protein activity. *J. Exp. Med.* **204,** 1119–1130 (2007).

151. Moro, K. *et al.* Innate production of T(H)2 cytokines by adipose tissue-associated c-Kit(+)Sca-1(+) lymphoid cells. *Nature* **463,** 540–4 (2010).

152. Yokota, Y. *et al.* Development of peripheral lymphoid organs and natural killer cells depends on the helix-loop-helix inhibitor Id2. *Nature* **397,** 702–706 (1999).

153. Diefenbach, A., Colonna, M. & Koyasu, S. Development , differentiation , and diversity of innate lymphoid cells. *Immunity* **41,** 354–365 (2014).

154. Cella, M., Miller, H. & Song, C. Beyond NK cells: The expanding universe of innate lymphoid cells. *Front. Immunol.* **5,** 1–11 (2014).

155. Yu, X. *et al.* The basic leucine zipper transcription factor NFIL3 directs the development of a common innate lymphoid cell precursor. *Elife* **22,** 1–20 (2014).

156. Seillet, C. *et al.* Nfil3 is required for the development of all innate lymphoid cell subsets. *J. Exp. Med.* **211,** 1733–40 (2014).

157. Geiger, T. L. *et al.* Nfil3 is crucial for development of innate lymphoid cells and host protection against intestinal pathogens. *J. Exp. Med.* **211,** 1723–1731 (2014).

158. Constantinides, M. G., McDonald, B. D., Verhoef, P. a & Bendelac, A. A committed precursor to innate lymphoid cells. *Nature* **508,** 397–401 (2014).

159. Klose, C. S. N. *et al.* Differentiation of type 1 ILCs from a common progenitor to all helper-like innate lymphoid cell lineages. *Cell* **157,** 340–356 (2014).

160. Verykokakis, M. *et al.* Essential functions for ID proteins at multiple checkpoints in invariant NKT cell development. *J. Immunol.* **191,** 5973–83 (2013).

161. Klein Wolterink, R. G. J. *et al.* Essential, dose-dependent role for the transcription factor Gata3 in the development of IL-5+ and IL-13+ type 2 innate lymphoid cells. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 10240–5 (2013).

162. Tindemans, I., Serafini, N., Di Santo, J. P. & Hendriks, R. W. GATA-3 function in innate and adaptive immunity. *Immunity* **41,** 191–206 (2014).

163. Rosmaraki, E. E. *et al.* Identification of committed NK cell progenitors in adult murine bone marrow. *Eur. J. Immunol.* **31,** 1900–1909 (2001).

164. Fathman, J., Bhattacharya, D., Inlay, M., Seita, J. & Weissman, I. Identification of the Earliest Committed Natural Killer Cell Progenitor in Murine Bone Marrow. *Clin. Immunol.* **118,** 5439–47 (2010).

165. Vosshenrich, C. a J. *et al.* Roles for common cytokine receptor gamma-chain-dependent cytokines in the generation, differentiation, and maturation of NK cell precursors and peripheral NK cells in vivo. *J. Immunol.* **174,** 1213–21 (2005).

166. Carotta, S., Pang, S. H. M., Nutt, S. L. & Belz, G. T. Identification of the earliest NK-cell precursor in the mouse BM. *Blood* **117,** 5449–5452 (2011).

167. Halim, T. Y. F. *et al.* Retinoic-acid-receptor-related orphan nuclear receptor alpha is required for natural helper cell development and allergic inflammation. *Immunity* **37,** 463–74 (2012).

168. Tang, Y. *et al.* Emergence of NK-cell progenitors and functionally competent NK-cell lineage subsets in the early mouse embryo. *Blood* **120,** 63–75 (2012).

169. Huntington, N. D., Vosshenrich, C. a J. & Di Santo, J. P. Developmental pathways that generate natural-killer-cell diversity in mice and humans. *Nat. Rev. Immunol.* **7,** 703–14 (2007).

170. Townsend, M. J. *et al.* T-bet regulates the terminal maturation and homeostasis of NK and Vα14i NKT cells. *Immunity* **20,** 477–494 (2004).

171. Gordon, S. M. *et al.* The Transcription Factors T-bet and Eomes Control Key Checkpoints of Natural Killer Cell Maturation. *Immunity* **36,** 55–67 (2012).

172. Bernink, J. H. *et al.* Human type 1 innate lymphoid cells accumulate in inflamed mucosal tissues. *Nat. Immunol.* **14,** 221–9 (2013).

173. Fuchs, A. *et al.* Intraepithelial type 1 innate lymphoid cells are a unique subset of IL-12- and IL-15-responsive IFN-γ-producing cells. *Immunity* **38,** 769–81 (2013).

174. Wong, S. H. *et al.* Transcription factor RORα is critical for nuocyte development. *Nat. Immunol.* **13,** 229–36 (2012).

175. Sawa, S. *et al.* Lineage relationship analysis of RORgammat+ innate lymphoid cells. *Science* **330,** 665–9 (2010).

176. Serafini, N. *et al.* Gata3 drives development of RORγt+ group 3 innate lymphoid cells. *J. Exp. Med.* **211,** 199–208 (2014).

177. Yagi, R. *et al.* The transcription factor GATA3 is critical for the development of all IL-7Rα-expressing innate lymphoid cells. *Immunity* **40,** 378–88 (2014).

178. Satoh-Takayama, N. *et al.* IL-7 and IL-15 independently program the differentiation of intestinal CD3-NKp46+ cell subsets from Id2-dependent precursors. *J. Exp. Med.* **207,** 273–80 (2010).

179. Cherrier, M., Sawa, S. & Eberl, G. Notch, Id2, and RORγt sequentially orchestrate the fetal development of lymphoid tissue inducer cells. *J. Exp. Med.* **209,** 729–40 (2012).

180. Spits, H. *et al.* Innate lymphoid cells--a proposal for uniform nomenclature. *Nat. Rev. Immunol.* **13,** 145–9 (2013).

181. Cella, M. *et al.* A human natural killer cell subset provides an innate source of IL-22 for mucosal immunity. *Nature* **457,** 722–725 (2009).

182. Scandella, E. *et al.* Restoration of lymphoid organ integrity through the interaction of lymphoid tissue-inducer cells with stroma of the T cell zone. *Nat. Immunol.* **9,** 667–675 (2008).

183. Monticelli, L. a *et al.* Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat. Immunol.* **12,** 1045–1054 (2011).

184. Abt, M. C. *et al.* Commensal Bacteria Calibrate the Activation Threshold of Innate Antiviral Immunity. *Immunity* **37,** 158–170 (2012).

185. Sonnenberg, G. F., Monticelli, L. a., Elloso, M. M., Fouser, L. a. & Artis, D. CD4+ Lymphoid Tissue-Inducer Cells Promote Innate Immunity in the Gut. *Immunity* **34,** 122–134 (2011).

186. Hanash, A. M. *et al.* Interleukin-22 protects intestinal stem cells from immune-mediated tissue damage and regulates sensitivity to graft versus host disease. *Immunity* **37,** 339–50 (2012).

187. Neill, D. R. *et al.* Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* **464,** 1367–1370 (2010).

188. Price, A. E. *et al.* Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 11489–94 (2010).

189. Sun, Z. *et al.* Requirement for RORgamma in thymocyte survival and lymphoid organ development. *Science* **288,** 2369–2373 (2000).

190. Van de Pavert, S. a & Mebius, R. E. New insights into the development of lymphoid tissues. *Nat. Rev. Immunol.* **10,** 664–74 (2010).

191. Cooper, M. a., Fehniger, T. a., Fuchs, A., Colonna, M. & Caligiuri, M. a. NK cell and DC interactions. *Trends Immunol.* **25,** 47–52 (2004).

192. Fehniger, T. a *et al.* CD56 bright natural killer cells are present in human lymph nodes and are activated by T cell – derived IL-2 : a potential new link between adaptive and innate immunity. *Blood* **101,** 3052–3057 (2003).

193. De Maria, A., Bozzano, F., Cantoni, C. & Moretta, L. Revisiting human natural killer cell subset function revealed cytolytic CD56 dim CD16 + NK cells as rapid producers of abundant IFN-γ on activation. *Proc. Natl. Acad. Sci. U. S. A.* **108,** 728–32 (2011).

194. Chan, A. *et al.* CD56bright human NK cells differentiate into CD56dim cells: role of contact with peripheral fibroblasts. *J. Immunol.* **179,** 89–94 (2007).

195. Romagnani, C. *et al.* CD56brightCD16- killer Ig-like receptor- NK cells display longer telomeres and acquire features of CD56dim NK cells upon activation. *J. Immunol.* **178,** 4947–4955 (2007).

196. Cooper, M. a *et al.* Human natural killer cells : a unique innate immunoregulatory role for the CD56 bright subset Human natural killer cells : a unique innate immunoregulatory role for the CD56 bright subset. *Blood* **97,** 3146–3151 (2001).

197. Hayakawa, Y. & Smyth, M. J. CD27 dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity. *J. Immunol.* **176,** 1517–1524 (2006).

198. Vosshenrich, C. a J. *et al.* A thymic pathway of mouse natural killer cell development characterized by expression of GATA-3 and CD127. *Nat. Immunol.* **7,** 1217–24 (2006).

199. Yu, J., Freud, A. G. & Caligiuri, M. a. Location and cellular stages of natural killer cell development. *Trends Immunol.* **34,** 573–582 (2013).

200. Yokoyama, W. A. M., SOjka, D. O. K., PEng, H. & Tian, Z. Tissue-Resident Natural Killer Cells. *Cold Spring Harb Symp Quant Biol* **LXXVIII,** (2013).

201. Tessmer, M. S., Reilly, E. C. & Brossay, L. Salivary gland NK cells are phenotypically and functionally unique. *PLoS Pathog.* **7,** 1–9 (2011).

202. Cortez, V. S., Fuchs, A., Cella, M., Gilfillan, S. & Colonna, M. Cutting edge: salivary gland NK cells develop independently of nfil3 in steady-state. *J. Immunol.* **192,** 4487–91 (2014).

203. Sojka, D. K. *et al.* Tissue-resident natural killer (NK) cells are cell lineages distinct from thymic and conventional splenic NK cells. *Elife* **2014,** 1–21 (2014).

204. Cheng, M. *et al.* Distinct and overlapping patterns of cytokine regulation of thymic and bone marrow-derived NK cell development. *J. Immunol.* **182,** 1460–1468 (2009).

205. Schulthess, J. *et al.* Interleukin-15-Dependent NKp46 + Innate Lymphoid Cells Control Intestinal Inflammation by Recruiting Inflammatory Monocytes. *Immunity* **37,** 108–121 (2012).

206. Daussy, C. *et al.* T-bet and Eomes instruct the development of two distinct natural killer cell lineages in the liver and in the bone marrow. *J. Exp. Med.* **211,** 563–77 (2014).

207. Cella, M., Otero, K. & Colonna, M. Expansion of human NK-22 cells with IL-7, IL-2, and IL-1beta reveals intrinsic functional plasticity. *Proc. Natl. Acad. Sci. U. S. A.* **107,** 10961–10966 (2010).

208. Klose, C. S. N. *et al.* A T-bet gradient controls the fate and function of CCR6-RORγt+ innate lymphoid cells. *Nature* **494,** 261–265 (2013).

209. Vonarbourg, C. *et al.* Regulated expression of nuclear receptor RORγt confers distinct functional fates to NK cell receptor-expressing RORγt(+) innate lymphocytes. *Immunity* **33,** 736–51 (2010).

210. Yang, Q. *et al.* T cell factor 1 is required for group 2 innate lymphoid cell generation. *Immunity* **38,** 694–704 (2013).

211. Drake, L. Y., Iijima, K. & Kita, H. Group 2 innate lymphoid cells and CD4+ T cells cooperate to mediate type 2 immune response in mice. *Allergy Eur. J. Allergy Clin. Immunol.* **69,** 1300–1307 (2014).

212. Turner, J.-E. *et al.* IL-9-mediated survival of type 2 innate lymphoid cells promotes damage control in helminth-induced lung inflammation. *J. Exp. Med.* **210,** 2951–65 (2013).

213. Neill, D. R. *et al.* Nuocytes represent a new innate effector leucocyte that mediates type-2 immunity. *Nature* **464,** 1367–1370 (2010).

214. Halim, T. Y. F., Krauss, R. H., Sun, A. C. & Takei, F. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. *Immunity* **36,** 451–63 (2012).

215. Imai, Y. *et al.* Skin-specific expression of IL-33 activates group 2 innate lymphoid cells and elicits atopic dermatitis-like inflammation in mice. *Proc. Natl. Acad. Sci. U. S. A.* **110,** 13921–13926 (2013).

216. Salimi, M. *et al.* A role for IL-25 and IL-33-driven type-2 innate lymphoid cells in atopic dermatitis. *J. Exp. Med.* **210,** 2939–50 (2013).

217. Fallon, P. G. *et al.* Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion. *J. Exp. Med.* **203,** 1105–16 (2006).

218. Walker, J. a. & McKenzie, A. N. J. Development and function of group 2 innate lymphoid cells. *Curr. Opin. Immunol.* **25,** 148–155 (2013).

219. Chang, Y.-J. *et al.* Innate lymphoid cells mediate influenza-induced airway hyper-reactivity independently of adaptive immunity. *Nat. Immunol.* **12,** 631–638 (2011).

220. Molofsky, A. B. *et al.* Innate lymphoid type 2 cells sustain visceral adipose tissue eosinophils and alternatively activated macrophages. *J. Exp. Med.* **210,** 535–49 (2013).

221. Nussbaum, J. C. *et al.* Type 2 innate lymphoid cells control eosinophil homeostasis. *Nature* **502,** 245–8 (2013).

222. Mjösberg, J. M. *et al.* Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. *Nat. Immunol.* **12,** 1055–62 (2011).

223. Cupedo, T. *et al.* Human fetal lymphoid tissue-inducer cells are interleukin 17-producing precursors to RORC+ CD127+ natural killer-like cells. *Nat. Immunol.* **10,** 66–74 (2009).

224. Sanos, S. L. *et al.* RORgammat and commensal microflora are required for the differentiation of mucosal interleukin 22-producing NKp46+ cells. *Nat. Immunol.* **10,** 83–91 (2009).

225. Satoh-Takayama, N. *et al.* Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. *Immunity* **29,** 958–70 (2008).

226. Takatori, H. *et al.* Lymphoid tissue inducer-like cells are an innate source of IL-17 and IL-22. *J. Exp. Med.* **206,** 35–41 (2009).

227. Kim, H. Y. *et al.* Interleukin-17-producing innate lymphoid cells and the NLRP3 inflammasome facilitate obesity-associated airway hyperreactivity. *Nat. Med.* **20,** 54–61 (2014).

228. Lee, J. S. *et al.* AHR drives the development of gut ILC22 cells and postnatal lymphoid tissues via pathways dependent on and independent of Notch. *Nat. Immunol.* **13,** 144–151 (2011).

229. Sciumé, G. *et al.* Distinct requirements for T-bet in gut innate lymphoid cells. *J. Exp. Med.* **209,** 2331–8 (2012).

230. Rankin, L. C. *et al.* The transcription factor T-bet is essential for the development of NKp46+ innate lymphocytes via the Notch pathway. *Nat. Immunol.* **14,** 389–95 (2013).

231. Buonocore, S. *et al.* Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. *Nature* **464,** 1371–5 (2010).

232. Robinette, M. L. *et al.* Transcriptional programs define molecular characteristics of innate lymphoid cell classes and subsets. *Nat. Immunol.* **16,** 306–17 (2015).

233. Hughes, T. *et al.* Stage 3 immature human natural killer cells found in secondary lymphoid tissue constitutively and selectively express the Th17 cytokine interleukin-22. *Blood* **113,** 4008–4010 (2009).

234. Satoh-Takayama, N. *et al.* The natural cytotoxicity receptor NKp46 is dispensable for IL-22-mediated innate intestinal immune defense against Citrobacter rodentium. *J. Immunol.* **183,** 6579–6587 (2009).

235. Dumoutier, L., Louahed, J. & Renauld, J. C. Cloning and characterization of IL-10-related T cell-derived inducible factor (IL-TIF), a novel cytokine structurally related to IL-10 and inducible by IL-9. *J. Immunol.* **164,** 1814–1819 (2000).

236. Wolk, K. *et al.* IL-22 increases the innate immunity of tissues. *Immunity* **21,** 241–254 (2004).

237. Longman, R. S. *et al.* CX3CR1+ mononuclear phagocytes support colitis-associated innate lymphoid cell production of IL-22. *J. Exp. Med.* **211,** 1571–1583 (2014).

238. Satoh-Takayama, N. *et al.* The Chemokine Receptor CXCR6 Controls the Functional Topography of Interleukin-22 Producing Intestinal Innate Lymphoid Cells. *Immunity* **41,** 776–788 (2014).

239. Zheng, Y. *et al.* Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat. Med.* **14,** 282–289 (2008).

240. Zhang, B. *et al.* Prevention and cure of rotavirus infection via TLR5/NLRC4 – mediated production of IL-22 and IL-18. *Science* **346,** 861–866 (2014).

241. Eken, a, Singh, a K., Treuting, P. M. & Oukka, M. IL-23R+ innate lymphoid cells induce colitis via interleukin-22-dependent mechanism. *Mucosal Immunol.* **7,** 143–54 (2014).

242. Huber, S. *et al.* IL-22BP is regulated by the inflammasome and modulates tumorigenesis in the intestine. *Nature* **491,** 259–263 (2012).

243. Kirchberger, S. *et al.* Innate lymphoid cells sustain colon cancer through production of interleukin-22 in a mouse model. *J. Exp. Med.* **210,** 917–31 (2013).

244. Chan, I. H. *et al.* Interleukin-23 is sufficient to induce rapid de novo gut tumorigenesis, independent of carcinogens, through activation of innate lymphoid cells. *Mucosal Immunol.* **7,** 842–856 (2014).

245. Geremia, A. *et al.* IL-23-responsive innate lymphoid cells are increased in inflammatory bowel disease. *J. Exp. Med.* **208,** 1127–33 (2011).

246. Villanova, F. *et al.* Characterization of innate lymphoid cells in human skin and blood demonstrates increase of NKp44+ ILC3 in psoriasis. *J. Invest. Dermatol.* **134,** 984–91 (2014).

247. Spencer, S. P. *et al.* Adaptation of Innate Lymphoid Cells to a Micronutrient Deficiency Promotes Type 2 Barrier Immunity. *Science* **343,** 432–7 (2014).

248. Luche, H. *et al.* In vivo fate mapping identifies pre-TCRα expression as an intra- and extrathymic, but not prethymic, marker of T lymphopoiesis. *J. Exp. Med.* **210,** 699–714 (2013).

249. Finotto, S. *et al.* Development of spontaneous airway changes consistent with human asthma in mice lacking T-bet. *Science* **295,** 336–338 (2002).

250. Gascoyne, D. M. *et al.* The basic leucine zipper transcription factor E4BP4 is essential for natural killer cell development. *Nat. Immunol.* **10,** 1118–1124 (2009).

251. Ginhoux, F. *et al.* Blood-derived dermal langerin+ dendritic cells survey the skin in the steady state. *J. Exp. Med.* **204,** 3133–3146 (2007).

252. Li, J. L. *et al.* Intravital multiphoton imaging of immune responses in the mouse ear skin. *Nat. Protoc.* **7,** 221–34 (2012).

253. Trichas, G., Begbie, J. & Srinivas, S. Use of the viral 2A peptide for bicistronic expression in transgenic mice. *BMC Biol.* **6,** 40 (2008).

254. Muroi, S. *et al.* Cascading suppression of transcriptional silencers by ThPOK seals helper T cell fate. *Nat. Immunol.* **9,** 1113–1121 (2008).

255. Cortes, P. *et al.* In vitro V(D)J recombination: signal joint formation. *Proc. Natl. Acad. Sci. U. S. A.* **93,** 14008–14013 (1996).

256. Ikawa, T. *et al.* An Essential Developmental Checkpoint for Production of the T Cell Lineage. *Science* **329,** 93–96 (2010).

257. Nitta, T., Ohigashi, I. & Takahama, Y. The Development of T Lymphocytes in Fetal Thymus Organ Culture. **946,** 85–102 (2013).

258. Mombaerts, P. *et al.* RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* **68,** 869–877 (1992).

259. Shinkai, Y. *et al.* RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* **68,** 855–867 (1992).

260. Russell, G. J. *et al.* Distinct structural and functional epitopes of the alpha E beta 7 integrin. *Eur. J. Immunol.* **24,** 2832–2841 (1994).

261. Cerf-Bensussan, N. *et al.* A monoclonal antibody (HML-1) defining a novel membrane molecule present on human intestinal lymphocytes. *Eur. J. Immunol.* **17,** 1279–1285 (1987).

262. Kilshaw, P. J. & Baker, K. C. A unique surface antigen on intraepithelial lymphocytes in the mouse. *Immunol. Lett.* **18,** 149–154 (1988).

263. Spits, H. & Di Santo, J. P. The expanding family of innate lymphoid cells: regulators and effectors of immunity and tissue remodeling. *Nat. Immunol.* **12,** 21–7 (2011).

264. Walker, J. a, Barlow, J. L. & McKenzie, A. N. J. Innate lymphoid cells--how did we miss them? *Nat. Rev. Immunol.* **13,** 75–87 (2013).

265. Flanagan, S. P. 'Nude', a new hairless gene with pleiotropic effects in the mouse. *Genet. Res.* **8,** 295–309 (1966).

266. Pantelouris, E. M. Absence of thymus in a mouse mutant. *Nature* **217,** 370–1 (1968).

267. Kondo, M. *et al.* Sharing of the interleukin-2 (IL-2) receptor gamma chain between receptors for IL-2 and IL-4. *Science* **262,** 1874–1877 (1993).

268. Miyazaki, T. *et al.* Functional activation of Jak1 and Jak3 by selective association with IL-2 receptor subunits. *Science* **266,** 1045–1047 (1994).

269. Noguchi, M. *et al.* Interleukin-2 receptor gamma chain: A functional component of the interleukin-7 receptor. *Science* **262,** 1877–1880 (1993).

270. Giri, J. G. *et al.* Utilization of the beta and gamma chains of the IL-2 receptor by the novel cytokine IL-15. *EMBO J.* **13,** 2822–2830 (1994).

271. Kimura, Y. *et al.* Sharing of the IL-2 receptor gamma chain with the functional IL-9 receptor complex. *Int. Immunol.* **7,** 115–120 (1995).

272. Russell, S. M. *et al.* Interleukin-2 receptor gamma chain: a functional component of the interleukin-4 receptor. *Science* **262,** 1880–1883 (1993).

273. Edelbaum, D., Mohamadzadeh, M., Bergstresser, P. R., Sugamura, K. & Takashima, a. Interleukin (IL)-15 promotes the growth of murine epidermal gamma delta T cells by a mechanism involving the beta- and gamma c-chains of the IL-2 receptor. *J. Invest. Dermatol.* **105,** 837–843 (1995).

274. Suzuki, H., Duncan, G. S., Takimoto, H. & Mak, T. W. Abnormal development of intestinal intraepithelial lymphocytes and peripheral natural killer cells in mice lacking the IL-2 receptor beta chain. *J. Exp. Med.* **185,** 499–505 (1997).

275. Lodolce, J. P. *et al.* IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation. *Immunity* **9,** 669–676 (1998).

276. Kennedy, M. K. *et al.* Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice. *J. Exp. Med.* **191,** 771–780 (2000).

277. Kawai, K. *et al.* Requirement of the IL-2 receptor chain for the development of Vgamma3 dendritic epidermal T cells. *J. Invest. Dermatol.* **110,** 961–965 (1998).

278. Honjo, M. *et al.* Thymus-independent generation of Thy-1+ epidermal cells from a pool of Thy-1- bone marrow precursors. *J. Invest. Dermatol.* **95,** 562–567 (1990).

279. Hoeffel, G. *et al.* Adult Langerhans cells derive predominantly from embryonic fetal liver monocytes with a minor contribution of yolk sac-derived macrophages. *J. Exp. Med.* **209,** 1167–81 (2012).

280. Ferrara, J. L. M. & Reddy, P. Pathophysiology of graft-versus-host disease. *Semin. Hematol.* **43,** 3–10 (2006).

281. Wilson, A., Capone, M. & MacDonald, H. R. Unexpectedly late expression of intracellular CD3epsilon and TCR gammadelta proteins during adult thymus development. *Int. Immunol.* **11,** 1641–50 (1999).

282. Levelt, C. N., Carsetti, R. & Eichmann, K. Regulation of thymocyte development through CD3. II. Expression of T cell receptor beta CD3 epsilon and maturation to the CD4+8+ stage are highly correlated in individual thymocytes. *J. Exp. Med.* **178,** 1867–75 (1993).

283. Smith, E. *et al.* T-lineage cells require the thymus but not VDJ recombination to produce IL-17A and regulate granulopoiesis in vivo. *J. Immunol.* **183,** 5685–93 (2009).

284. Von Boehmer, H. Unique features of the pre-T-cell receptor alpha-chain: not just a surrogate. *Nat. Rev. Immunol.* **5,** 571–577 (2005).

285. Bruno, L., Rocha, B., von Boehmer, H. & Rodewald, H.-R. Intra- and extra-thymic expression of the pre-T cell receptor alpha gene. *Eur. J. Immunol.* **25,** 1877–1882 (1995).

286. Luche, H., Weber, O., Rao, T. N., Blum, C. & Fehling, H. J. Faithful activation of an extra-bright red fluorescent protein in 'knock-in' Cre-reporter mice ideally suited for lineage tracing studies. *Eur. J. Immunol.* **37,** 43–53 (2007).

287. Dudley, E. C., Girardi, M., Owen, M. J. & Hayday, a C. Alpha beta and gamma delta T cells can share a late common precursor. *Curr Biol* **5,** 659–669 (1995).

288. Aifantis, I. *et al.* On the Role of the Pre-T Cell Receptor in alpha beta versus gamma delta T Lineage Commitment. *Immunity* **9,** 649–655 (1998).

289. Wilson, A. & MacDonald, H. R. A limited role for beta-selection during gamma delta T cell development. *J. Immunol.* **161,** 5851–5854 (1998).

290. Seibler, J. *et al.* Rapid generation of inducible mouse mutants. *Nucleic Acids Res.* **31,** e12 (2003).

291. Rothenberg, E. V, Moore, J. E. & Yui, M. a. Launching the T-cell-lineage developmental programme. *Nat. Rev. Immunol.* **8,** 9–21 (2008).

292. Germain, R. N. T-cell development and the CD4-CD8 lineage decision. *Nat. Rev. Immunol.* **2,** 309–22 (2002).

293. Leclercq, G., Plum, J., Nandi, D., Smedt, M. De & Allison, J. P. Intrathymic differentiation of Vgamma3 T cells. *J. Exp. Med.* **178,** 309–315 (1993).

294. Havran, W. L. & Allison, J. P. Developmentally ordered appearance of thymocytes expressing different T-cell antigen receptors. *Nature* **335,** 443–5 (1988).

295. Watanabe, Y. *et al.* A murine thymic stromal cell line which may support the differentiation of CD4-8- thymocytes into CD4+8- alpha beta T cell receptor positive T cells. *Cell. Immunol.* **142,** 385–397 (1992).

296. Douagi, I., Vieira, P. & Cumano, A. Lymphocyte commitment during embryonic development , in the mouse. *Semin. Immunol.* **14,** 361–369 (2002).

297. Jenkinson, E. J., Franchi, L. L., Kingston, R. & Owen, J. J. Effect of deoxyguanosine on lymphopoiesis in the developing thymus rudiment in vitro: application in the production of chimeric thymus rudiments. *Eur. J. Immunol.* **12,** 583–587 (1982).

298. Biron, C. a, Young, H. a & Kasaian, M. T. Interleukin 2-induced proliferation of murine natural killer cells in vivo. *J. Exp. Med.* **171,** 173–188 (1990).

299. Ishida, B. Y. Y., Nishi, M., Taguchi, O., Kawaichi, M. & Honjo, T. Expansion of Natural Killer Cells but not T cells in Human Interleukin2/Interleukin 2 Receptor (Tac) Transgenic Mice. *J. Exp. Med.* **170,** 1103–15 (1989).

300. Philips, J. H., Takeshita, T., Sugamuraj, K. & Lanier, L. L. Activation of Natural Killer Cells via the p75 Interleukin 2 Receptor. *J. Exp. Med.* **170,** (1989).

301. Garni-Wagner, B. A., Purohit, A., Mathew, P. A., Bennett, M. & Kumar, V. A novel function-associated molecule related to non-MHC-restricted cytotoxicity mediated by activated natural killer cells and T cells. *J. Immunol.* **151,** 60–70 (1993).

302. Schuhmachers, G. *et al.* 2B4, a new member of the immunoglobulin gene superfamily, is expressed on murine dendritic epidermal T cells and plays a functional role in their killing of skin tumors. *J. Invest. Dermatol.* **105,** 592–596 (1995).

303. Mathew, P. a *et al.* Cloning and characterization of the 2B4 gene encoding a molecule associated with non-MHC-restricted killing mediated by activated natural killer cells and T cells. *J. Immunol.* **151,** 5328–5337 (1993).

304. Brown, M. H. *et al.* 2B4, the natural killer and T cell immunoglobulin superfamily surface protein, is a ligand for CD48. *J. Exp. Med.* **188,** 2083–2090 (1998).

305. Ozawa, H., Aiba, S., Nakagawa, S. & Tagami, H. Murine epidermal Langerhans cells express CD48, which is a counter-receptor for mouse CD2. *Arch. Dermatol. Res.* **287,** 524–528 (1995).

306. Chaix, J. *et al.* Cutting edge: Priming of NK cells by IL-18. *J. Immunol.* **181,** 1627–1631 (2008).

307. Ponti, C. *et al.* Role of CREB transcription factor in c-fos activation in natural killer cells. *Eur. J. Immunol.* **32,** 3358–3365 (2002).

308. Levanon, D. *et al.* Transcription factor Runx3 regulates interleukin-15-dependent natural killer cell activation. *Mol Cell Biol* **34,** 1158–1169 (2014).

309. Seillet, C. *et al.* Differential requirement for Nfil3 during NK cell development. *J. Immunol.* **192,** 2667–76 (2014).

310. Kamizono, S. *et al.* Nfil3/E4bp4 is required for the development and maturation of NK cells in vivo. *J. Exp. Med.* **206,** 2977–2986 (2009).

311. Blauvelt, a *et al.* Interleukin-15 mRNA is expressed by human keratinocytes Langerhans cells, and blood-derived dendritic cells and is downregulated by ultraviolet B radiation. *J. Invest. Dermatol.* **106,** 1047–1052 (1996).

312. Mohamadzadeh, M. *et al.* Interleukin 15 skews monocyte differentiation into dendritic cells with features of Langerhans cells. *J. Exp. Med.* **194,** 1013–1020 (2001).

313. Rückert, R. *et al.* Inhibition of keratinocyte apoptosis by IL-15: a new parameter in the pathogenesis of psoriasis? *J. Immunol.* **165,** 2240–2250 (2000).

314. Havran, W. L. & Allison, J. P. Origin of Thy-1+ dendritic epidermal cells of adult mice from fetal thymic precursors. *Nature* **344,** 68–70 (1990).

315. Rocha, B. The extrathymic T-cell differentiation in the murine gut. *Immunol. Rev.* **215,** 166–77 (2007).

316. Allen, S., Turner, S. J., Bourges, D., Gleeson, P. a & van Driel, I. R. Shaping the T-cell repertoire in the periphery. *Immunol. Cell Biol.* **89,** 60–69 (2011).

317. Chatterjea-Matthes, D. *et al.* Early defect prethymic in bone marrow T cell progenitors in athymic nu/nu mice. *J. Immunol.* **171,** 1207–1215 (2003).

318. Merad, M. *et al.* Langerhans cells renew in the skin throughout life under steady-state conditions. *Nat. Immunol.* **3,** 1135–1141 (2002).

319. Lee, K. *et al.* 2B4 (CD244)/ CD48 in the generation of NK effector functions Requirement of homotypic NK-cell interactions through 2B4 (CD244)/ CD48 in the generation of NK effector functions. *Sci. Technol.* **107,** 3181–3188 (2011).

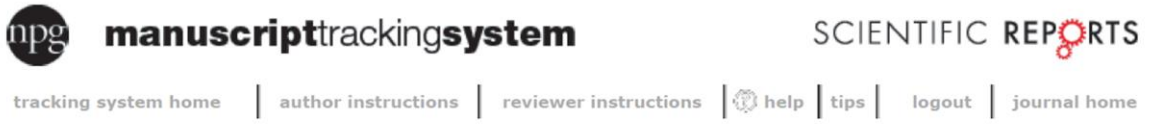
320. Schoenborn, J. R. & Wilson, C. B. Regulation of Interferon-gamma During Innate and Adaptive Immune Responses. *Adv. Immunol.* **96,** 41–101 (2007).

321. Naik, S. *et al.* Commensal–dendritic-cell interaction specifies a unique protective skin immune signature. *Nature* **520,** 104–8 (2015).

ANNEXES

8. Annexes

Almeida, FF *et al*, Identification of a transient T cell progenitor-like lymphoid population in the murine epidermis. Submitted to *Scientific Reports* (7th April 2015)



Detailed Status Information

Manuscript #	SREP-15-07233
Current Revision #	0
Submission Date	7th April 15
Current Stage	Manuscript Submitted
Title	Identification of a transient T cell progenitor-like lymphoid population in the murine epidermis
Manuscript Type	Original Research - FAST TRACK TRIAL
Manuscript Comment	
Corresponding Author	Prof. Florent Ginhoux (florent_ginhoux@immunol.a-star.edu.sg) (Agency for Science, Technology and Research (A*STAR))
Contributing Authors	Mrs. Francisca F Almeida , Dr. Mari Tenno , Dr. Joanna Brzostek , Dr. Jackson LiangYao Li , Dr. Guillaume Hoeffel , Mr. Peter See , Dr. Lai Guan Ng , Prof. Hans Jörg Fehling , Prof. Nicholas R. J. Gascoigne , Prof. Ichiro Taniuchi
Authorship	Yes
Abstract	T cell progenitors are known to arise from the foetal liver in embryos and the bone marrow in adults; however different studies have shown that a pool of T cell progenitors may also exist in the periphery. Here, we identified a lymphoid population resembling peripheral T cell progenitors which transiently seed the epidermis during late embryogenesis in both wild-type and T cell-deficient mice. We named these cells ELCs (epidermal lymphoid cells). ELCs expressed Thy1 and CD2, but lacked CD3 and TCRαβ/γδ at their surface, reminiscent of the phenotype of extra- or intra- thymic T cell progenitors. Similarly to dendritic epidermal T cells (DETCs), ELCs were radio-resistant and capable of self-renewal. However, despite their progenitor-like phenotype and expression of T cell lineage markers within the population, ELCs did not differentiate into conventional T cells or DETCs in in vitro, ex vivo or in vivo differentiation assays. Therefore we report the discovery of a unique population of lymphoid cells within the murine epidermis that appears to represent a distinct sub-population of immune cells with as-yet-unidentified origin, nature and functions.
Techniques	Life sciences techniques [Cell/tissue technologies]; Life sciences techniques, Cell/tissue technologies [Flow cytometry];
Subject Terms	Biological sciences/Immunology Biological sciences/Immunology/Lymphocytes/T cells
Competing Financial Interest	There is **NO** Competing Interest.
Applicable Funding Source	No Applicable Funding

Stage	Start Date	End Date	Approximate Duration
Manuscript Submitted	7th April 15		
Author Approved Converted Files	7th April 15		
Preliminary Manuscript Data Submitted	7th April 15		